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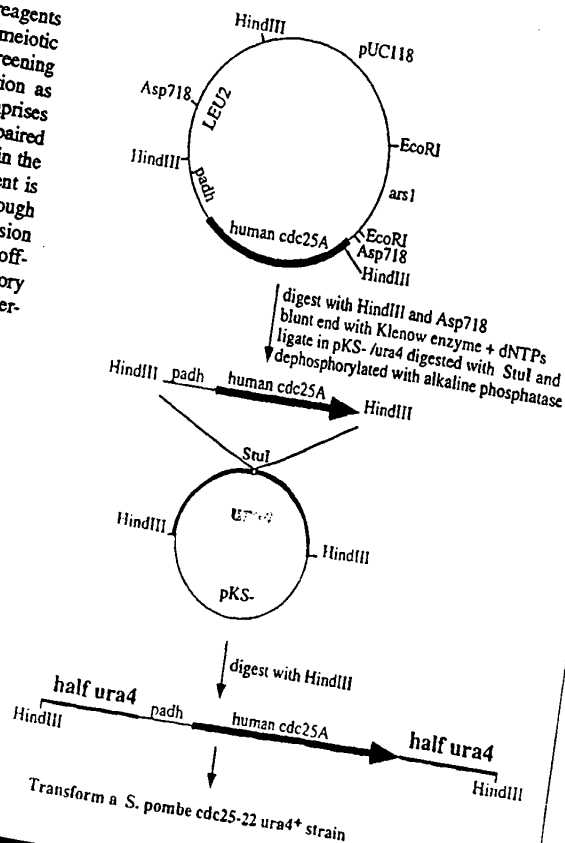
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(54) Title: ASSAY AND REAGENTS FOR IDENTIFYING ANTI-PROLIFERATIVE AGENTS

(57) Abstract

The present invention makes available assays and reagents for identifying antiproliferative agents, such as mitotic and meiotic inhibitors. The present assay provides a simple and rapid screening test which relies on scoring for positive cellular proliferation as indicative of anti-mitotic or anti-meiotic activity, and comprises contacting a candidate agent with a cell which has an impaired cell-cycle checkpoint and measuring the level of proliferation in the presence and absence of the agent. The checkpoint impairment is such that it either causes premature progression of the cell through at least a portion of a cell-cycle or inhibition of normal progression of the cell through at least a portion of a cell-cycle, but can be offset by the action of an agent which inhibits at least one regulatory protein of the cell cycle (e.g., cdc25) in a manner which counterbalances the effect of the impairment.



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Assay and Reagents for Identifying Anti-proliferative Agents

Background of the Invention

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Entry of cells into mitosis characteristically involves coordinated and simultaneous events, which include, for example, cytoskeletal rearrangements, disassembly of the nuclear envelope and of the nucleoli, and condensation of chromatin into chromosomes. Cell-cycle events are thought to be regulated by a series of interdependent biochemical steps, with the
10 initiation of late events requiring the successful completion of those proceeding them. In eukaryotic cells mitosis does not normally take place until the G1, S and G2 phases of the cell-cycle are completed. For instance, at least two stages in the cell cycle are regulated in response to DNA damage, the G1/S and the G2/M transitions. These transitions serve as checkpoints to which cells delay cell-cycle progress to allow repair of damage before entering
15 either S phase, when damage would be perpetuated, or M phase, when breaks would result in loss of genomic material. Both the G1/S and G2/M checkpoints are known to be under genetic control as there are mutants that abolish arrest or delay which ordinarily occur in wild-type cells in response to DNA damage.

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The progression of a proliferating eukaryotic cell through the cell-cycle checkpoints is controlled by an array of regulatory proteins that guarantee that mitosis occurs at the appropriate time. These regulatory proteins can provide exquisitely sensitive feedback-controlled circuits that can, for example, prevent exit of the cell from S phase when a fraction of a percent of genomic DNA remains unreplicated (Dasso et al. (1990) *Cell* 61:811-823) and
25 can block advance into anaphase in mitosis until all chromosomes are aligned on the metaphase plate (Rieder et al. (1990) *J. Cell Biol.* 110:81-95). In particular, the execution of various stages of the cell-cycle is generally believed to be under the control of a large number of mutually antagonistic kinases and phosphatases. For example, genetic, biochemical and morphological evidence implicate the cdc2 kinase as the enzyme responsible for triggering
30 mitosis in eukaryotic cells (for reviews, see Hunt (1989) *Curr. Opin. Cell Biol.* 1:268-274; Lewin (1990) *Cell* 61:743-752; and Nurse (1990) *Nature* 344:503-508). The similarities between the checkpoints in mammalian cells and yeast have suggested similar roles for cdc protein kinases across species. In support of this hypothesis, a human cdc2 gene has been found that is able to substitute for the activity of an *S. Pombe* cdc2 gene in both its G1/S and
35 G2/M roles (Lee et al (1987) *Nature* 327:31). Likewise, the fact that the cdc2 homolog of *S. Cerevisiae* (cdc28) can be replaced by the human cdc2 also emphasizes the extent to which the basic cell-cycle machinery has been conserved in evolution.

As mitosis progresses, the cdc2 kinase appears to trigger a cascade of downstream

mitotic phenomena such as metaphase alignment of chromosomes, segregation of sister chromatids in anaphase, and cleavage furrow formation. Many target proteins involved in mitotic entry of the proliferating cell are directly phosphorylated by the cdc2 kinase. For instance, the cdc2 protein kinase acts by phosphorylating a wide variety of mitotic substrates such as nuclear lamins, histones, and microtubule-associated proteins (Moreno et al. (1990) *Cell* 61:549-551; and Nigg (1991) *Semin. Cell Biol.* 2:261-270). The cytoskeleton of cultured cells entering mitosis is rearranged dramatically. Caldesmon, an actin-associated protein, has also been shown to be a cdc2 kinase substrate (Yamashiro et al. (1991) *Nature* 349:169-172), and its phosphorylation may be involved in induction of M-phase-specific dissolution of actin cables. The interphase microtubule network disassembles, and it replaced by a mitosis-specific astral array emanating from centrosomes. This rearrangement has been correlated with the presence of mitosis-specific cdc2 kinase activity in cell extracts (Verde et al (1990) *Nature* 343:233-238). Changes in nuclear structure during mitotic entry are also correlated with cdc2 kinase activity. Chromatin condensation into chromosomes is accompanied by cdc2 kinase-induced phosphorylation of histone H1 (Langan et al. (1989) *Molec. Cell. Biol.* 9:3860-3868), nuclear envelope dissolution is accompanied by cdc2-specific phosphorylation of lamin B (Peter et al. (1990) *Cell* 61:591-602) nucleolar disappearance is coordinated with the cdc2-dependent phosphorylation of nucleolin and NO38.

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The activation of cdc2 kinase activity occurs during the M phase and is an intricately regulated process involving the concerted binding of an essential regulatory subunit (i.e., a cyclin) and phosphorylation at multiple, highly conserved positions (for review, see Fleig and Gould (1991) *Semin. Cell Biol.* 2:195-204). The complexity of this activation process most likely stems from the fact that, as set out above, the initiation of mitosis must be keyed into a number of signal transduction processes whose function is to guard against the inappropriate progression of the cell-cycle. In particular, the cell employs such signaling mechanisms to guarantee that mitosis and cytokinesis do not occur unless cellular growth and genome duplication have occurred in an accurate and timely manner.

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The cdc2 kinase is subject to multiple levels of control. One well-characterized mechanism regulating the activity of cdc2 involves the phosphorylation of tyrosine, threonine, and serine residues; the phosphorylation level of which varies during the cell-cycle (Draetta et al. (1988) *Nature* 336:738-744; Dunphy et al. (1989) *Cell* 58:181-191; Morla et al. (1989) *Cell* 58:193-203; Gould et al. (1989) *Nature* 342:39-45; and Solomon et al. (1990) *Cell* 63:1013-1024). The phosphorylation of cdc2 on Tyr-15 and Thr-14, two residues located in the putative ATP binding site of the kinase, negatively regulates kinase activity.

This inhibitory phosphorylation of cdc2 is mediated at least in part by the wee1 and mik1 tyrosine kinases (Russell et al. (1987) *Cell* 49:559-567; Lundgren et al. (1991) *Cell* 64:1111-1122; Featherstone et al. (1991) *Nature* 349:808-811; and Parker et al. (1992) *PNAS* 89:2917-2921). These kinases act as mitotic inhibitors, over-expression of which causes cells to arrest in the G2 phase of the cell-cycle. By contrast, loss of function of wee1 causes a modest advancement of mitosis, whereas loss of both wee1 and mik1 function causes grossly premature mitosis, uncoupled from all checkpoints that normally restrain cell division (Lundgren et al. (1991) *Cell* 64:1111-1122).

As the cell is about to reach the end of G2, dephosphorylation of the cdc2-inactivating Thr-14 and Tyr-15 residues occurs leading to activation of the cdc2 complex as a kinase. A stimulatory phosphatase, known as cdc25, is responsible for Tyr-15 and Thr-14 dephosphorylation and serves as a rate-limiting mitotic activator. (Dunphy et al. (1991) *Cell* 67:189-196; Lee et al. (1992) *Mol Biol Cell* 3:73-84; Millar et al. (1991) *EMBO J* 10:4301-4309; and Russell et al. (1986) *Cell* 45:145-153). Recent evidence indicates that both the cdc25 phosphatase and the cdc2-specific tyrosine kinases are detectably active during interphase, suggesting that there is an ongoing competition between these two activities prior to mitosis (Kumagai et al. (1992) *Cell* 70:139-151; Smythe et al. (1992) *Cell* 68:787-797; and Solomon et al. (1990) *Cell* 63:1013-1024). This situation implies that the initial decision to enter mitosis involves a modulation of the equilibrium of the phosphorylation state of cdc2 which is likely controlled by variation of the rate of tyrosine dephosphorylation of cdc2 and/or a decrease in the rate of its tyrosine phosphorylation. A variety of genetic and biochemical data appear to favor a decrease in cdc2-specific tyrosine kinase activity near the initiation of mitosis which can serve as a triggering step to tip the balance in favor of cdc2 dephosphorylation (Smythe et al. (1992) *Cell* 68:787-797; Matsumoto et al. (1991) *Cell* 66:347-360; Kumagai et al. (1992) *Cell* 70:139-151; Rowley et al. (1992) *Nature* 356:353-355; and Enoch et al. (1992) *Genes Dev.* 6:2035-2046). Moreover, recent data suggests that the activated cdc2 kinase is responsible for phosphorylating and activating cdc25. This event would provide a self-amplifying loop and trigger a rapid increase in the activity of the cdc25 protein, ensuring that the tyrosine dephosphorylation of cdc2 proceeds rapidly to completion (Hoffmann et al. (1993) *EMBO J.* 12:53).

Summary of the Invention

The present invention makes available assays and reagents for identifying anti-proliferative agents, such as mitotic and meiotic inhibitors. The present assay provides a simple and rapid screening test which relies on scoring for positive cellular proliferation as indicative of anti-mitotic or anti-meiotic activity, and comprises contacting a candidate agent with a cell which has an impaired cell-cycle checkpoint and measuring the level of proliferation in the presence and absence of the agent. The checkpoint impairment is such that it either causes premature progression of the cell through at least a portion of a cell-cycle or inhibition of normal progression of the cell through at least a portion of a cell-cycle, but can be off-set by the action of an agent which inhibits at least one regulatory protein of the cell-cycle in a manner which counter-balances the effect of the impairment. In one embodiment of the assay, anti-mitotic agents can be identified through their ability to rescue an otherwise hyper-mitotic cell from mitotic catastrophe (e.g. cell death) by inhibiting the activity of at least one regulatory protein of the cell-cycle which acts as a mitotic activator. In another embodiment of the assay, an anti-mitotic agent can be identified by its ability to induce mitosis in an otherwise hypo-mitotic cell by inhibiting the activity of at least one regulatory protein of the cell-cycle which acts as a negative regulator of mitosis. In yet another embodiment of the invention, anti-meiotic agents can be identified by their ability to bring about faithful meiosis of an otherwise hyper-meiotic or hypo-meiotic cell.

The impaired checkpoint can be generated, for example, by molecular biological, genetic, and/or biochemical means. The checkpoint to be impaired can comprise a regulatory protein or proteins which control progression through the cell-cycle, such as those which control the G2/M transition or the G1/S transition. By way of example, the impaired checkpoint can comprise regulatory proteins which control the phosphorylation/dephosphorylation of a cdc protein kinase, such as the gene products of *wee1*, *mik1*, or *nim1*.

The cell used in the assay (reagent cell) can be generated so as to favor scoring for anti-proliferative agents which specifically inhibit a particular cell-cycle activity. For example, if it is desirable to produce an inhibitor to a *cdc25* phosphatase activity, a hyper-mitotic or hyper-meiotic cell can be generated which would be rescued from mitotic or meiotic catastrophe by partial inhibition of *cdc25*.

Furthermore, the hyper- and hypo-proliferative cells of the present assay, whether for identifying anti-mitotic or anti-meiotic agents, can be generated so as to comprise heterologous cell-cycle proteins (i.e. cross-species expression). For example, a *cdc25* homolog from one species can be expressed in the cells of another species where it has been

shown to be able to rescue loss-of-function mutations in that host cell. For example, a hypermitotic *Schizosaccharomyces* cell, such as *Schizosaccharomyces pombe*, can be constructed so as to comprise an exogenous *cdc25* phosphatase and a conditionally impairable *wee1* protein kinase. The exogenous *cdc25* can be, for example, a human *cdc25* homolog, or
5 alternatively, a *cdc25* homolog from a human pathogen.

Description of the Drawings

Figure 1 is a schematic representation of the construction of the "5'-half *ura4*-*adh*
10 promoter- *cdc25A*-3'-half *ura4*" nucleic acid fragment of Example 1 for transforming *ura4*+
S. pombe cells.

Figure 2 is a schematic representation of the construction of the "5'-half *ura4*-*adh*
15 promoter- *cdc25B*-3'-half *ura4*" nucleic acid fragment of Example 2 for transforming *ura4*+ *S.*
pombe cells.

Figure 3 is a schematic representation of the construction of the pART3-*cdc25C*
plasmid of Example 3.

Figure 4 is a schematic representation of the construction of the "5'-half *ura4*-*adh*
20 promoter- *cdc25C*-3'-half *ura4*" nucleic acid fragment of Example 3 for transforming *ura4*+ *S.*
pombe cells.

Figure 5A and 5B are photographs of yeast colonies formed by *S. pombe* cells
25 transformed with pART3 plasmid, grown at 25°C and 37°C respectively.

Figures 6A and 6B are photographs of yeast colonies formed by *S. pombe* cells
transformed with the pARTN-*cdc25A* plasmid of Example 1, grown at 25°C and 37°C
respectively.

30 Figures 7A and 7B are photographs of yeast colonies formed by *S. pombe* cells
transformed with the pARTN-*cdc25B* plasmid of Example 1, grown at 25°C and 37°C
respectively.

35 Figures 8A and 8B are photographs of yeast colonies formed by *S. pombe* cells
transformed with the pARTN-*cdc25C* plasmid of Example 1, grown at 25°C and 37°C
respectively.

Detailed Description of the Invention

In dividing eukaryotic cells, circuits of regulatory proteins oversee both the initiation and completion of the major transitions of both the meiotic and mitotic cell-cycles. These regulatory networks guarantee that the successive events of each cell-cycle occur in a faithful and punctual manner. For example, mitosis cannot begin until the cell has grown sufficiently and replicated its genome accurately. Likewise, cell division cannot ensue until the mitotic spindle has distributed the chromosomes equally to both daughter cells.

The present invention makes available assays and reagents for identifying anti-mitotic and anti-meiotic agents. As described herein, anti-mitotic agents can be identified, in one embodiment of the present assay, through their ability to rescue an otherwise hyper-mitotic cell from mitotic catastrophe by inhibiting the activity of at least one regulatory protein of the cell-cycle which acts as a mitotic activator. The term hyper-mitotic cell denotes a cell having an impaired cell-cycle checkpoint which can cause premature progression of the cell through at least a portion of the cell-cycle and thereby results in inhibition of proliferation of the cell. The impaired checkpoint of the hyper-mitotic cell would otherwise act as a negative regulator of downstream mitotic events. Impairment of such a negative regulator consequently allows the cell to proceed aberrantly toward subsequent mitotic stages and ultimately inhibits faithful proliferation of the cell. In the presence of an agent able to inhibit a mitotic activator, progression of the hyper-mitotic cell through the cell-cycle can be slowed to enable the cell to appropriately undergo mitosis and proliferate with fidelity. In general, it will be expected that in order to detect an anti-mitotic agent in the present assay using a hyper-mitotic cell, the agent must inhibit a mitotic activator whose operation in the cell-cycle is sufficiently connected to the impaired checkpoint that the cell is prevented by the anti-mitotic agent from committing to the otherwise catastrophic events of prematurely passing the checkpoint. It is clear that an anti-mitotic agent effective at rescuing the hyper-mitotic cell in the present assay can do so by acting directly on the mitotic activator such as, for example, a phosphatase inhibitor might be expected to do to a cdc25 homolog. Alternatively, the anti-mitotic agent may exert its effect by preventing the activation of the mitotic activator, as, for example, inhibiting the phosphorylation step which activates cdc25 as a phosphatase, or inhibiting the activity of the cdc2 kinase with regard to other potential protein substrates.

In another embodiment of the present assay, an anti-mitotic agent can be identified by its ability to induce mitosis in an otherwise hypo-mitotic cell by inhibiting the activity of at least one regulatory protein of the cell-cycle which acts as a negative regulator of mitosis. The term hypo-mitotic cell refers to a cell which has an impaired checkpoint comprising an overly-active negative mitotic regulator which represses progression of the cell through at

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least a portion of the cell-cycle. In the presence of an agent able to inhibit the activity of the negative regulator, inhibition of the cell-cycle is overcome and the cell can proliferate at an increased rate relative to the untreated hypo-mitotic cell. As with the hyper-mitotic system above, it will generally be expected that an anti-mitotic agent detected in the hypo-mitotic system acts at, or sufficiently close to, the overly-active negative regulator so as to reduce its inhibitory effect on the cell-cycle.

In yet another embodiment of the present invention, anti-meiotic agents can be identified in a manner analogous to the anti-mitotic assay above, wherein faithful meiosis of either a hyper-meiotic or hypo-meiotic cell is measured in the presence and absence of a candidate agent. As above, the terms hyper-meiotic and hypo-meiotic refer to impaired meiotic checkpoints which are respectively of either diminished activity or enhanced activity relative to the normal meiotic cell.

The present assay provides a simple and rapid screening test which relies on scoring for positive proliferation as indicative of anti-mitotic activity. One advantage of the present assay is that while direct inhibition of growth can be caused by any toxic compound added to a proliferating cell culture, growth stimulation in the present assay will only be achieved upon specific inhibition of a mitotic activator where the assay comprises a hyper-mitotic cell, or upon inhibition of a negative mitotic regulator where the assay comprises a hypo-mitotic cell. In an analogous manner, positive meiotic progression can be utilized in the present assay as indicative of anti-meiotic activity of the candidate agent.

Other advantages of the present assays include the ability to screen for anti-mitotic and anti-meiotic activity *in vivo*, as well as the amenity of the assay to high through-put analysis. Anti-mitotic agents identified in the present assay can have important medical consequences and may be further tested for use in treating proliferative diseases which include a wide range of cancers, neoplasias, and hyperplasias, as well as for general or specific immunosuppression, such as through inhibition of the proliferation of lymphocytes. In addition, the present assay can be used to identify both anti-mitotic and anti-meiotic agents which can be used in the treatment of pathogenic infections such as fungal infections which give rise to mycosis. Anti-mitotic and anti-meiotic agents identified in the present assay may also be used, for example, in birth control methods by disrupting oogenic pathways in order to prevent the development of either the egg or sperm, or by preventing mitotic progression of a fertilized egg.

With regard to the hyper-mitotic cell and hypo-mitotic cell of the present assay,

impairment of the negative regulatory checkpoint can be generated so as to be either continual or conditional. A conditional impairment permits the checkpoint to be normatively operational under some conditions such that the cell may proliferate and be maintained by cell culture techniques; and be rendered inoperative, or alternatively hyper-operative, under other conditions. In the instance of the hyper-mitotic cell, the impaired checkpoint is effectively inoperative to an extent that the impairment allows aberrant mitosis to occur which concludes in mitotic catastrophe (e.g. cell death). Conversely, the hypo-mitotic cell can be generated by an impaired checkpoint which is effectively hyper-operative and results in inhibition of the cell-cycle. A continual impairment, on the other hand, is one that is ever-present and which allows proliferation of the cell under conditions where there is no need to halt the cell at that checkpoint; but, in the instance of the hyper-mitotic cell, results in mitotic catastrophe under conditions where the cell-cycle must be halted, such as in the presence of DNA synthesis inhibitors or DNA damaging agents.

The impaired checkpoint can be generated, for example, by molecular biological, genetic, and/or biochemical means. The checkpoint to be impaired can comprise a regulatory protein or proteins which control progression through the cell-cycle, such as those which control the G2/M transition or the G1/S transition. Extensive genetic and biochemical analysis of these pathways (see, for example, *Molecular Biology of the Fission Yeast*, eds Nasi et al., Academic Press, San Diego, 1989) has led to the ability to manipulate the control of mitosis through loss-of-function and gain-of-function mutations and by plasmid overexpression, as well as by exposure of the cell to certain chemicals. The checkpoint impairment can be, for example, the result of directly altering the effective activity of a regulatory protein at the checkpoint (i.e. by altering its catalytic activity and/or concentration), or indirectly the result of modifying the action of another protein which is upstream of the checkpoint but which modulates the action of regulatory proteins at the checkpoint. For instance, various mutants have been isolated which are able to escape specific cell-cycle control circuits and progress inappropriately to the next cell-cycle stage and can be used to generate the hyper-mitotic cell. In a similar manner, mutants have been isolated which are unable to pass a specific cell-cycle checkpoint and are prevented from progressing to the next cell-cycle stage, and provide the basis for the hypo-mitotic cell of the present assay.

Genetic studies in eukaryotic systems, including mammalian and fungi, have identified several genes that are important for the proper timing of mitosis. For instance, in the fission yeast *S. pombe*, genes encoding regulators of cell division have been extensively characterized (for review see MacNeil et al. (1989) *Curr. Genet.* 16:1). As set out above,

initiation of mitosis in fission yeast correlates with activation of the cdc2 protein kinase. cdc2 is a component of M phase promoting factor (MPF) purified from frogs and starfish, and homologs of cdc2 have been identified in a wide range of eukaryotes, suggesting that cdc2 plays a central role in mitotic control in all eukaryotic cells (Norbury et al. (1989) *Biochem. Biophys. Acta* 989:85). For purposes of the present disclosure, the term "cdc2" or "cdc protein kinase" is used synonymously with the recently adopted "cyclin-dependent kinase" (cdk) nomenclature. Furthermore as used herein, the term cdc2 is understood to denote members of the cyclin-dependent kinase (cdk) family. Representative examples of cdc protein kinases include cdc2-SP, cdc28 (*S. Cerevisiae*), cdk2-XL, cdc2-HS and cdk2-HS, where "HS" designates *homosapiens*, SP designates *S. pombe*, and "XL" designates *Xenopus Laevis*. As set out above, the switch that controls the transition between the inactive cdc2/cyclin B complex (phosphorylated on Try-15 and Thr-14) present during S-G2-prophase and the active form of the cdc2/cyclin B kinase (dephosphorylated on Try-15 and Thr-14) present at metaphase is believed to correspond to a change in the relative activities of the opposing kinases and phosphatase(s) that act on the sites. Given that many regulatory pathways appear to converge on cdc protein kinases, as well as their activating role at both G1/S and G2/M transitions, the hyper-mitotic cell of the present assay can be employed to develop inhibitors specific for particular cdc protein kinases.

Regulatory pathways which feed into and modulate the activity of a cdc protein kinase can be manipulated to generate either the hyper-mitotic or hypo-mitotic cell of the present assay. For example, the inhibitory phosphorylation of cdc2 is mediated by at least two tyrosine kinases, initially identified in fission yeast and known as weel and mik1 (Russell et al. (1987) *Cell* 49:559; Lundgren et al. (1991) *Cell* 64:111; Featherstone et al. (1991) *Nature* 349:808; and Parker et al. (1991) *EMBO* 10:1255). These kinases act as mitotic inhibitors, overexpression of which causes cells to arrest in the G2 phase of the cell-cycle. For instance, overexpression of weel has been shown to cause intense phosphorylation of cdc2 (cdc28 in budding yeast) which results in cell-cycle arrest. Conversely, loss of function of weel causes advancement of mitosis and cells enter mitosis at approximately half the normal size, whereas loss of weel and mik1 function causes grossly premature initiation of mitosis, uncoupled from all checkpoints that normally restrain cell division. Thus, weel and mik1 each represent suitable regulatory proteins which could be impaired to generate either the hyper-mitotic or hypo-mitotic cell of the present assay.

Furthermore, it is apparent that enzymes which modulate the activity of the weel or mik1 kinases can also be pivotal in controlling the precise timing of mitosis. For example, the level of the nim1/cdr1 protein, a negative regulator of the weel protein kinase, can have a

pronounced impact on the rate of mitotic initiation, and *nim1* mutants have been shown to be defective in responding to nutritional deprivation (Russel et al. (1987) *Cell* 49:569; and Feilotter et al. (1991) *Genetics* 127:309). Over-expression of *nim1* (such as the *S. pombe* op-
5 *nim1* mutant) can result in inhibition of the *wee1* kinase and allow premature progression into mitosis. Loss of *nim1* function, on the other hand, delays mitosis until the cells have grown to a larger size. In like manner, mutation in the *stf1* gene has also been shown to relieve regulation of mitotic progression in response to DNA synthesis inhibition.

Loss-of-function strains, such as *wee1-50*, *mik1::ura*, or *stf1-1* (Rowley et al. (1992)
10 *Nature* 356:353), are well known. In addition, each of the *wee1*, *mik1*, and *nim1* genes have been cloned (see for example Coleman et al. (1993) *Cell* 72:919; and Feilotter et al. (1991) *Genetics* 127:309), such that disruption of *wee1* and/or *mik1* expression or over-expression of *nim1* can be carried out to create the hyper-mitotic cell of the present assay. In a similar
15 fashion, over-expression of *wee1* and/or *mik1* or disruption of *nim1* expression can be utilized to generate the hypo-mitotic cell of the present assay. Furthermore, each of these negative mitotic regulators can also be a potential target for an anti-mitotic agent scored for using the hypo-mitotic cell of the present assay.

Acting antagonistically to the *wee1/mik1* kinases, genetic and biochemical studies
20 have indicated that the *cdc25* protein is a central player in the process of *cdc2*-specific dephosphorylation and crucial to the activation of the *cdc2* kinase activity. In the absence of *cdc25*, *cdc2* accumulates in a tyrosine phosphorylated state and can cause inhibition of mitosis. The phosphatase activity of *cdc25* performs as a mitotic activator and is therefore a suitable target for inhibition by an anti-mitotic agent in the present assay. It is strongly
25 believed that this aspect of the mitotic control network is generally conserved among eukaryotes, though the particular mode of regulation of *cdc25* activity may vary somewhat from species to species. Homologs of the fission yeast *cdc25* have been identified in the budding yeast *S. cerevisiae* (Millar et al. (1991) *CSH Symp. Quant. Biol.* 56:577), humans (Galaktinov et al. (1990) *Cell* 67:1181; and Sadhu et al. (1989) *PNAS* 87:5139), mouse
30 (Kakizuka et al. (1992) *Genes Dev.* 6:578), *Drosophila* (Edgar et al. (1989) *Cell* 57:177; and Glover (1991) *Trends Genet.* 7:125), and *Xenopus* (Kumagai et al., (1992) *Cell* 70:139; and Jessup et al. (1992) *Cell* 68:323). Human *cdc25* is encoded by a multi-gene family now consisting of at least three members, namely *cdc25A*, *cdc25B* and *cdc25C*. As described below, all three homologs are able to rescue temperature-sensitive mutations of the *S. Pombe*
35 *cdc25*. Early evidence suggests that these different homologs may have different functions. For instance, microinjection of anti-*cdc25-C* antibodies into mammalian cells prevents them from dividing. They appear to arrest in interphase with a flattened morphology, consistent

with a role for cdc25C in the entry into mitosis. On the contrary, microinjection of antibodies to cdc25A results in a rounded-up mitotic-like state, suggesting that the different homologs may have distinct functions and represent an additional level of complexity to the control of M-phase onset by cdc25 in higher eukaryotes. Comparison of the human cdc25's with each other and with cdc25 homologs from other species has been carried out. Comparison of cdc25A with cdc25C demonstrates a 48% identity in the 273 C-terminal region between the two proteins; and comparison between cdc25B and cdc25C reveals a 43% identity. The *Drosophila* cdc25 homolog "string" shares 34.5% identity to cdc25A in a 362 amino acid region and 43.9% in an 269 amino acid region with cdc25B. *S. Pombe* cdc25 is also related to the human cdc25's, but to a lesser extent. Interestingly, the overall similarity between different human cdc25 proteins does not greatly exceed that between humans and such evolutionary distinct species as *Drosophila*. Biochemical experiments have demonstrated that bacterially produced cdc25 protein from *Drosophila* and human activates the histone H1 kinase activity of cdc2 in *Xenopus* or starfish extracts (Kumagai et al. (1991) *Cell* 64:903; and Strausfield et al. (1991) *Nature* 351:242).

If the cdc25 phosphatase activity is the desired target for development of an anti-mitotic agent, it may be advantageous to choose the hyper-mitotic cell of the present assay so as to more particularly select for anti-mitotic agents which act directly or indirectly on cdc25. As set out above, it will generally be expected that in order to score for an anti-mitotic agent in an assay relying on a hyper-mitotic cell, the inhibited mitotic activator (e.g. cdc25) must be sufficiently connected to the abherent checkpoint so as to rescue the cell before it concludes in mitotic catastrophe. Furthermore, the hyper-mitotic cell of the present assay can be generated by manipulation of the cell in which a cdc25 homolog is endogenously expressed, as for example, by generating a *wee1* mutation (a "wee" phenotype), or by exposure of the cell to 2-aminopurine or caffeine after a γ -radiation induced G2 arrest. Alternatively, the cdc25 gene from one species or cell type can be cloned and subsequently expressed in a cell to which it is not endogenous but in which it is known to rescue lack-of-function mutations of the endogenous cdc25 activity. For example, the exogenous cdc25, such as a human cdc25, could be expressed in an hyper-mitotic *Schizosaccharomyces* cell, such as an *S. pombe* cell like the temperature-sensitive *wee1-50* mutant. It may be possible to take advantage of the structural and functional differences between the human cdc25 phosphatases to provide anti-mitotic agents which selectively inhibit particular human cell types. In a similar manner, it may be feasible to develop cdc25 phosphatase inhibitors with the present assay which act specifically on pathogens, such as fungus involved in mycotic infections, without substantially inhibiting the human homologs.

The cdc2 activating kinase (CAK) represents yet another potential target for inhibition by an anti-mitotic agent which could be scored for using the hyper-mitotic cell of the present assay. Recent evidence indicates that many, if not all, of the cdc protein kinases require cyclin binding as well as phosphorylation at Thr-161 (Thr-161 of cdc2-HS; Thr-167 of cdc-2SP; Thr-169 of cdc28; and Thr-160 of cdk2-HS) for activation *in vivo*. CAK is believed to direct phosphorylation of Thr-161 in a cyclin-dependent manner and to act as a mitotic activator. Inhibition of CAK by a candidate agent may offset the effect of a hyper-mitotic checkpoint impairment which would otherwise have led to premature activation of a cdc protein kinase (e.g. as a wee1 deficient mutant would). In addition, CAK itself represents a possible site of impairment to generate the hyper-mitotic cell of the present assay. Overexpression of CAK can lead to premature activation of a cdc protein kinase and cause the cell to conclude in mitotic catastrophe.

Other checkpoints which could be impaired to generate the hyper-mitotic and hypomitotic systems have been identified by examination of mitotic events in cells treated in a manner which disrupts DNA synthesis or DNA repair. Radiation-induced arrest is one example of a checkpoint mechanism which has been used to identify both negative and positive regulators of mitosis. In this instance, mitosis is delayed until the integrity of the genome is checked and, as far as possible, restored. Checkpoint controls also function to delay mitosis until DNA synthesis is complete. The observation of cell-cycle arrest points indicate that the regulation of progression into mitosis in response to both DNA damage and the DNA synthesis requires components of the mitotic control. For example, analysis of radiation-sensitive mutations in budding yeast have identified a number of defective regulatory proteins which can prevent the arrest of the cell-cycle in response to DNA damage and are therefore potential candidates for impairment to generate the hyper-mitotic or hypomitotic cell of the present assay. By way of illustration, a number of genes involved in this mitotic feedback control have been identified, and include the rad9, rad17, rad24, mec1, mec2 and mec3 genes (Weinert et al. (1988) *Science* 241:317). All six genes have been shown to be negative regulators of cell-cycle progression and act in response to damaged DNA. Two genes, mec1 and mec2, are also involved in arresting the cell-cycle in response to unreplicated DNA.

The response to DNA damage has also been investigated in the fission yeast *S. pombe*. Mutations in a number of genes have been identified which allow cells with damaged or unreplicated DNA to enter mitosis. For example, the HUS12 and HUS16 genes have been implicated as negative regulators of mitosis which respond to unreplicated DNA, while RAD21 is a negative regulator sensitive to damaged DNA. The HUS14, HUS17, HUS22,

HUS26, RAD1, RAD3, RAD9 and RAD17 genes of *S. Pombe* each appear to be negative regulators of mitosis which are able to respond to either unreplicated or damaged DNA. (Rowley et al. (1992) *EMBO* 11:1343; and Enoch et al (1991) *CSH Symp. Quant. Biol.* 56:409)

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Recently, mutations in the *S. cerevisiae* genes BUB and MAD have been isolated which fail to arrest in mitosis with microtubule-destabilizing drugs. (Hayt et al. (1991) *Cell* 66:507; and Li et al. (1991) *Cell* 66:519). The *S. cerevisiae* cell can also be affected by a number of environmental cues. One such effector is the α -mating factor which induces G1 arrest. Mutants in the FUS3 or FAR1 genes fail to arrest in G1 in response to α -factor. While mutations in either gene are phenotypically similar, they affect different regulatory pathways. For example, the FUS3 gene has been cloned and exhibits strong sequence similarity to the serine/threonine family of protein kinases (Goebel et al. (1991) *Curr. Opin. Cell Biol.* 3:242).

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In the fungus *Aspergillus nidulans*, the bimE gene is believed to code for a negative regulator of mitosis that normally functions to prevent mitosis by controlling expression of a putative mitotic inducer, nimA. The absence of bimE function is believed to override cell-cycle control systems normally operative to prevent chromosome condensation and spindle formation from occurring during interphase. Temperature sensitive mutants of the bimE gene, such as the bimE7 mutant, allow cells with unreplicated DNA to prematurely enter mitosis (Osmani et al. (1988) *Cell* 52:241) and can be lethal phenotypes useful as hyper-mitotic cells of the present assay.

25

Checkpoints, and mutations thereof, have been identified in mammalian cells as well, and can be used to generate the hyper-mitotic and hypo-mitotic cells of the present assay. For instance, uncoupling of mitosis from completion of DNA replication has been reported in mammalian cells in response to drug treatment and mutation. In mammalian cells, as in other eukaryotic cells, DNA damage caused by mild X-ray irradiation can block passage through two cell-cycle checkpoints, the restriction point (G1/S) and entry into mitosis (G2/M) (Little et al. (1968) *Nature* 218:1064; Nagasawa et al. (1984) *Radiation Res.* 97:537; and Murray (1992) *Nature* 359:599). The AT gene(s), p53 and GADD45 are among genes which have been identified as critical to negative regulation of mitosis by cell-cycle checkpoints (Kaastan et al. (1992) *Cell* 71:587; Hartwell (1992) *Cell* 71:543; and Murray (1992) *Nature* 359:599) and can be utilized in the present assay to generate a hyper-mitotic cell or a hypo-mitotic cell depending on whether the impairment is brought about by disruption of expression, inhibition of activity, or by overexpression. Additionally, a temperature-sensitive mutation in the

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mammalian RCC1 (repressor of chromosome condensation) gene can cause cultured hamster cells to cease DNA replication and enter mitosis prematurely when they are shifted up to the nonpermissive temperature during S. phase. Relatives of RCC1 have also been identified in yeast (i.e. pim1) and *Drosophila*, and both genes can complement the mammalian RCC1 mutation, further suggesting that certain checkpoint mechanisms, like cdc2 regulation of the cell-cycle, are conserved across diverse phyla.

Many of the regulatory proteins involved in the progression of a cell through meiosis have also been identified. Because of the commonality of certain mitotic and meiotic pathways, several mitotic regulatory proteins or their homologs, such as cdc protein kinases, cyclins, and cdc25 homologs, also serve to regulate meiosis. For example, cell division cycle mutants defective in certain mitotic cell-cycle events have been tested for sporulation at semi-restrictive temperatures (Gralbert et al. (1991) *Curr Genet* 20:199). The mitotic defective mutants cdc10-129, cdc20-M10, cdc21-M6B, cdc23-M36 and cdc24-M38 formed four-spored asci but with low efficiency. Mutants defective in the mitotic initiation genes cdc2, cdc25 and cdc13 were blocked at meiosis II, though none of the wee1-50, ddh. nim1+ and win1+ alleles had any affect on sporulation, suggesting that their interactions with cdc25 and cdc2 are specific to mitosis in yeast. Other regulatory genes and gene products which can be manipulated to form the hyper- or hypo-meiotic cells of the present invention include rec102, spo13, cut1, cut2, IME1, MAT, RME1, cdc35, BCY1, TPK1, TPK2, TPK3, spd1, spd3, spd4, spo50, spo51, and spo53. As above, the hyper- or hypo-meiotic cells can be generated genetically or chemically using cells to which the intended target of the anti-meiotic agent is endogenous, or alternatively, using cells in which the intended target is exogenously expressed.

In addition, certain meiotic regulatory proteins are able to rescue loss-of-function mutations in the mitotic cell-cycle. For example, the *Drosophila* meiotic cdc25 homolog, "twine", is able to rescue mitosis in temperature-sensitive cdc25 mutants of fission yeast. Thus, anti-meiotic agents can be identified using hyper- or hypo-meiotic cells, and in some instances, hyper- or hypo-mitotic cells.

It is also deemed to be within the scope of this invention that the hyper- and hypo-proliferative cells of the present assay, whether for identifying anti-mitotic or anti-meiotic agents, can be generated so as to comprise heterologous cell-cycle proteins (i.e. cross-species expression). As exemplified above in the instance of cdc25, cell-cycle proteins from one species can be expressed in the cells of another and have been shown to be able to rescue

loss-of-function mutations in the host cell. In addition to those cell-cycle proteins which are ideally to be the target of inhibition by the candidate agent, cell-cycle proteins which interact with the intended inhibitor target can also be expressed across species. For example, in an hyper-proliferative yeast cell in which a human cdc25 (e.g. exogenously expressed) is the intended target for development of an anti-mitotic agent, a human cdc protein kinase and human cyclin can also be expressed in the yeast cell. Likewise, when a hypo-proliferative yeast expressing human wee1 is used, a human cdc protein kinase and human cyclin with which the human cdc25 would interact can be used to replace the corresponding yeast cell-cycle proteins. To illustrate, a triple *cln* deletion mutant of *S. Cerevisiae* which is also conditionally deficient in *cdc28* (the budding yeast equivalent of *cdc2*) can be rescued by the co-expression of a human cyclin and human *cdc2* proteins, demonstrating that yeast cell-cycle machinery can be at least in part replaced with corresponding human regulatory proteins. Roberts et al. (1993) *PCT Publication Number WO 93/06123*. In this manner, the reagent cells of the present assay can be generated to more closely approximate the natural interactions which a particular cell-cycle protein might experience.

Manipulation of these regulatory pathways with certain drugs, termed here "hyper-mitotic agents", can induce mitotic aberrations and result in generation of the hyper-mitotic cell of the present assay. For instance, caffeine, the protein kinase inhibitors 2-aminopurine and 6-dimethylaminopurine, and the protein phosphatase inhibitor okadaic acid can cause cells that are arrested in S phase by DNA synthesis inhibitors to inappropriately enter mitosis (Schlegel et al. (1986) *Science* 232:1264; Schlegel et al. (1987) *PNAS* 84:9025; and Schlegel et al. (1990) *Cell Growth Differ.* 1:171). Further, 2-aminopurine is believed to be able to override a number of cell-cycle checkpoints from G1, S phase, G2, or mitosis. (Andreassen et al. (1992) *PNAS* 89:2272; Andreassen et al. (1991) *J. Cell Sci.* 100:299, and Steinmann et al. (1991) *PNAS* 88:6843). For example, 2-aminopurine permits cells to overcome a G2/M block induced by γ -irradiation. Additionally, cells continuously exposed to 2-aminopurine alone are able to exit S phase without completion of replication, and exit mitosis without metaphase, anaphase, or telophase events.

In an analogous manner, hypo-mitotic agents, such as a phosphatase inhibitor, can be utilized to chemically induce impairment of one or more regulatory pathways to produce the hypo-mitotic cell of the present assay. Likewise, hyper-meiotic or hypo-meiotic agents can be employed to chemically generate the appropriate reagent cell for identifying anti-meiotic agents in the present assay.

To aid in the facilitation of mitotic catastrophe in the hyper-mitotic cell it may be

desirable to expose the cell to an agent (i.e. a chemical or environmental stimulus) which ordinarily induces cell-cycle arrest at that checkpoint. Inappropriate exit from the chemically- or environmentally-induced arrested state due to the impairment of the negative regulatory checkpoint can ultimately be lethal to the cell. Such arresting agents can include

5 exposure to DNA damaging radiation or DNA damaging agents; inhibition of DNA synthesis and repair using DNA polymerase inhibitors such as hydroxyurea or aphidicolin; topoisomerase inhibitors such as 4'-dimethyl-epipodophyllotoxin (VM-26); or agents which interfere with microtubule-assembly, such as Nocadazole and taxol. By way of example, BHK and HeLa cells which receive 250 rads of γ radiation have been shown to undergo G2

10 arrest that was reversed without further treatment within 4-5 hours. However, in the presence of either caffeine, 2-aminopurine, or 6-dimethyl-aminopurine, this mitotic delay was suppressed in both the hamster and human cells, and allowed the cells undergo mitosis before DNA repair had been completed (Steinmann et al. (1991) *PNAS* 88:6843). Additionally, in certain cells, nutritional status of the cell, as well as mating factors, can cause arrest of the

15 normal cell during mitosis.

The present assay can be used to develop inhibitors of fungal infections. The most common fungal infections are superficial and are presently treated with one of several topical drugs or with the oral drugs ketoconazole or griseofulvin. The systemic mycoses constitute

20 quite a different therapeutic problem. These infections are often very difficult to treat and long-term, parenteral therapy with potentially toxic drugs may be required. The systemic mycoses are sometimes considered in two groups according to the infecting organism. The "opportunistic infections" refer to those mycoses -candidiasis, aspergillosis, cryptococcosis, and phycomycosis- that commonly occur in debilitated and immunosuppressed patients.

25 These infections are a particular problem in patients with leukemias and lymphomas, in people who are receiving immunosuppressive therapy, and in patients with such predisposing factors as diabetes mellitus or AIDS. Other systemic mycoses -for example, blastomycosis, histoplasmosis, coccidioidomycosis, and sporotrichosis- tend to have a relatively low incidence that may vary considerably according to geographical area.

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To develop an assay for anti-mitotic or anti-meiotic agents having potential therapeutic value in the treatment of a certain mycotic infection, a yeast implicated in the infection can be used to generate the appropriate reagent cell of the present assay. For example, the hyper-mitotic or hypo-mitotic cell can be generated biochemically as described

35 above, or engineered, as for example, by screening for radiation-sensitive mutants having impaired checkpoints. Additionally, a putative mitotic regulator of the mycotic yeast, such as a *cdc25* homolog, can be cloned and expressed in a heterologous cell which may be easier to

manipulate or facilitate easier measurement of proliferation, such as member of the *Schizosaccharomyces* genus like *S. pombe*.

By way of illustration, the present assays can be used to screen for anti-mitotic and anti-meiotic agents able to inhibit at least one fungus implicated in such mycosis as candidiasis, aspergillosis, mucormycosis, blastomycosis, geotrichosis, cryptococcosis, chromoblastomycosis, coccidioidomycosis, conidiosporosis, histoplasmosis, maduromycosis, rhinosporidiosis, nocardiosis, para-actinomycosis, penicilliosis, monoliasis, or sporotrichosis. For example, if the mycotic infection to which treatment is desired is candidiasis, the present assay can comprise either a hyper-mitotic or hypo-mitotic cells generated directly from, or with genes cloned from, yeast selected from the group consisting of *Candida albicans*, *Candida stellatoidea*, *Candida tropicalis*, *Candida parapsilosis*, *Candida krusei*, *Candida pseudotropicalis*, *Candida quilliermondii*, and *Candida rugosa*. Likewise, the present assay can be used to identify anti-mitotic and anti-meiotic agents which may have therapeutic value in the treatment of aspergillosis by making use of yeast such as *Aspergillus fumigatus*, *Aspergillus flavus*, *Aspergillus niger*, *Aspergillus nidulans*, or *Aspergillus terreus*. Where the mycotic infection is mucormycosis, the yeast can be selected from a group consisting of *Rhizopus arrhizus*, *Rhizopus oryzae*, *Absidia corymbifera*, *Absidia ramosa*, and *Mucor pusillus*. Other pathogens which can be utilized in the present assay include *Pneumocystis carinii* and *Toxoplasma gondii*.

Agents to be tested for their ability to act as anti-mitotic and/or anti-meiotic agents in the present assay can be those produced by bacteria, yeast or other organisms, or those produced chemically. The assay can be carried out in any vessel suitable for the growth of the cell, such as microtitre plates or petri dishes. As potent inhibitors mitosis and/or meiosis can fully inhibit proliferation of a cell, it may be useful to perform the assay at various concentrations of the candidate agent. For example, serial dilutions of the candidate agents can be added to the hyper-mitotic cell such that at at least one concentration tested the anti-mitotic agent inhibits the mitotic activator to an extent necessary to adequately slow the progression of the cell through the cell-cycle but not to the extent necessary to inhibit entry into mitosis all together. In a like manner, where the assay comprises a hypo-mitotic cell, serial dilutions of a candidate agent can be added to the cells such that, at at least one concentration, an anti-mitotic agent inhibits a negative mitotic regulator to an extent necessary to adequately enhance progression of the cell through the cell-cycle, but not to an extent which would cause mitotic catastrophe.

Quantification of proliferation of the hyper-mitotic cell in the presence and absence of a candidate agent can be measured with a number of techniques well known in the art,

including simple measurement of population growth curves. For instance, where the assay involves proliferation in a liquid medium, turbidimetric techniques (i.e. absorbance/transmittance of light of a given wavelength through the sample) can be utilized. For example, in the instance where the reagent cell is a yeast cell, measurement of absorbance
5 of light at a wavelength between 540 and 600nm can provide a conveniently fast measure of cell growth.

Likewise, ability to form colonies in solid medium (e.g. agar) can be used to readily score for proliferation. Both of these techniques, especially with respect to yeast cells, are suitable for high through-put analysis necessary for rapid screening of large numbers of
10 candidate agents. In addition, the use of solid media such as agar can further aid in establishing a serial dilution of the candidate agent. For example, the candidate agent can be spotted on a lawn of reagent cells plated on a solid media. The diffusion of the candidate agent through the solid medium surrounding the site at which it was spotted will create a diffusional effect. For anti-mitotic or anti-meiotic agents scored for in the present assay, a
15 halo of cell growth would be expected in an area which corresponds to concentrations of the agent which offset the effect of the impaired checkpoint, but which are not so great as to over-compensate for the impairment or too little so as to be unable to rescue the cell.

To further illustrate, other proliferative scoring techniques useful in the present assay include measuring the mitotic index for untreated and treated cells; uptake of detectable
20 nucleotides, amino acids or dyes; as well as visual inspection of morphological details of the cell, such as chromatin structure or other features which would be distinguishable between cells advancing appropriately through mitosis and cells concluding in mitotic catastrophe or stuck at certain cell-cycle checkpoint. In the instance of scoring for meiosis, morphology of the spores or gametes can be assessed. Alternatively, the ability to form a viable spore of
25 gamete can be scored as, for example, measuring the ability of a spore to re-enter negative growth when contacted with an appropriate fermentable media.

To test compounds that might specifically inhibit the human *cdc25A*, *cdc25B* or *cdc25C* gene products, the genes were introduced into the genome of an *S. pombe* strain
30 which was engineered to be conditionally hyper-mitotic. Three linear DNA fragments were constructed, each carrying one of the three human *cdc25A*, *B* or *C* genes under the control of an *S. pombe* promoter, and flanked by nucleic acid sequences which allow integration of the DNA into the *S. pombe* genome. The *cdc25*-containing DNA fragments are then used to transform an appropriate *S. pombe* strain. For example, in one embodiment, the expression of
35 the human *cdc25* gene is driven by the strong *adh* promoter and the flanking sequences of the fragment contain the *ura4* gene to allow integration of the fragment at the *ura4* locus by

homologous recombination (Grimm et al. (1988) *Molec. gen. Genet* 81-86). The *S. pombe* strain is a weel temperature-sensitive mutant which becomes hyper-mitotic at temperatures above 36 °C, and carries a wild-type ura4 gene in which the cdc25 DNA fragment can be integrated.

5

Example 1

The human cdc25A gene has been previously cloned (see Galaktinov et al. (1991) *Cell* 67:1181). The sequence of the cdc25A gene containing the open reading frame is shown in Seq. ID No. 1, and is predicted to encode a protein of 523 amino acids (Seq. ID No. 2). A 2.0 kb NcoI-KpnI fragment encoding amino acids 1-523 of human cdc25A was subcloned into a NcoI-KpnI-(partially) digested pARTN expression vector, resulting in the pARTN-cdc25A construct harboring human cdc25A cDNA in sense orientation to the constitutive *adh* promoter. The *S. Pombe* autonomously replicating pARTN vector is derived from pART3 (McLeod et al. (1987) *EMBO* 6:729) by ligation of a NcoI linker (New England Biolabs) into the SmaI site.

20 A 2.3 kb DNA fragment corresponding to the *adh* promoter and amino acids 1-523 of the human cdc25A gene, was isolated by digesting the pARTN-cdc25A plasmid with HindIII and Asp718. While HindIII is sufficient to isolate the *adh* promoter/human cdc25A gene fragment from the plasmid, we also used Asp718 to cut the close migrating 2.2 kb HindIII-HindIII *S. cerevisiae* LEU2 gene in two smaller fragments which makes isolation of the cdc25A fragment easier.

25 The HindIII/HindIII fragment was then blunt ended with Klenow enzyme and dNTPs (see *Molecular Cloning: A Laboratory Manual 2ed*, eds. Sambrook et al., CSH Laboratory Press: 1989) and ligated into a pKS-ura4 plasmid previously digested with StuI and dephosphorylated with alkaline phosphatase. Massive amounts of the recombinant plasmid were prepared, and a 4.1 kb DNA fragment corresponding to "5'-half ura4-*adh* promoter-cdc25A-3'-half ura4" (see Figure 1) was isolated.

30

Example 2

35 The human cdc25B gene has been previously cloned (see Galaktinov et al. (1991) *Cell* 67:1181). The sequence of the cdc25B gene containing the open reading frame is shown in Seq. ID. No. 3, and is predicted to encode a protein of 566 amino acids (Seq. ID No. 4). A 2.4 kb SmaI fragment from the p4x1.2 plasmid (Galaktinov et al., *supra*) encoding amino

- acids 32-566 was subcloned into a SmaI-digested pART3 vector, resulting in the pARTN-cdc25B vector containing the human cdc25B cDNA. While the site of initiation of translation is not clear (there is no exogenous ATG 5' to the SmaI cloning site in the cdc25B open reading frame) we speculate that the first ATG corresponds to the Met-59 of the human cdc25B open reading frame, or alternatively, an ATG at an NdeI site of pART3. In any event, the pARTN-cdc25B plasmid has been shown to be capable of transforming *S. pombe* cells and able to rescue temperature-sensitive mutations of the yeast cdc25 gene (Galaktinov et al., supra).
- 10 As above, a 2.7 kb DNA fragment, corresponding to the adh promoter and amino acids 32-566 of the human cdc25B gene, was isolated by digesting pARTN-cdc25B with HindIII and Asp718. The HindIII/HindIII cdc25B fragment was blunt ended with Klenow enzyme and dNTPs, and ligated into a pKS-/ura4 vector previously digested with StuI and dephosphorylated with alkaline phosphatase. A 4.4 kb DNA fragment corresponding to "5'-half ura4-adh promoter-cdc-25B-3'-half ura4" (see Figure 2) was isolated.

Example 3

- The human cdc25C gene has been previously cloned (see Sadhu et al. (1990) *PNAS* 87:115139; and Hoffmann et al. (1993) *EMBO* 12:53). The sequence of the cdc25C gene containing the open reading frame is shown in Seq. ID No. 5, and is predicted to encode a protein of 473 amino acids (Seq. ID No. 6). Beginning with the pGEX-2T6-cdc25 plasmid (Hoffmann et al., supra) a 1.8 kbp DNA fragment corresponding to amino acids 1-473 of the human cdc25C gene was isolated digestion with BamHI and by partial digestion with NdeI (i.e., there is a NdeI site in the cdc25C gene). This fragment was ligated into a pART3 vector previously digested with NdeI and BamHI, resulting in the plasmid pART3-cdc25C which contained the amino acids 1-473 of the human cdc25C gene under the control of the strong adh promoter (see Figure 3).
- 30 A 2.5 kbp fragment corresponding to the adh promoter and amino acids 1-473 of the human cdc25C gene was isolated by digesting pART3-cdc25C with HindIII and Asp718. The HindIII/HindIII cdc25C fragment was blunt ended with Klenow enzyme and dNTPs, and ligated into a pKS-/ura4 plasmid previously digested with StuI and dephosphorylated with alkaline phosphatase. A 4.3 kbp DNA fragment corresponding to "5'-half ura4-adh promoter-cdc25C-3'-half ura4" (see Figure 4) was isolated.

Example 4

Each of the *cdc25* plasmid constructs pARTN-*cdc25A*, pARTN-*cdc25B*, and pART3-*cdc25C*, as well as the original pART3 plasmid, were used to transform the *S. Pombe* strain Sp553 (h+N, *cdc25-22*, *wee1-50*, *leul-32*) using well known procedures. Briefly, cells were grown in YE medium at 25°C until they were in exponential phase (~10⁷ cells/ml). The cells were then spun down from the media at 3000rpm for 5 minutes, and resuspended in LiCl/TE at a concentration of ~10⁸ cells/ml (LiCl/TE=10mM Tris, 1mM EDTA, 50 mM LiCl, Ph 8). The resuspended cells were incubated at room temperature for 10 minutes, then spun again at 3000rpm for 5 minutes, resuspended in LiCl/TE to a concentration of ~5 x 10⁸ cells/ml, and shaken for 30 minutes at 25°C.

To an aliquot of 150µl of cells, 500 ng of plasmid DNA and 350µL of PEG/TE (10mM Tris, 1mM EDTA, 50% PEG 4000, Ph 8) was added. The cell/plasmid mixture was then incubated for 30 minutes at 25°C, heat shocked at 42°C for 20 minutes, then spun at 15,000 rpm for 10 seconds after the addition of 0.5 mL of EMM. The cells were resuspended in 0.6 mL EMM, and 0.2 mL aliquots were plated.

Figures 5A and 5B illustrate the ability of the pART3 transformed yeast to grow at 25°C and 37°C respectively. As set out above, at the non-permissive temperature of 37°C, both the endogenous *wee1* and *cdc25* activities are impaired such that they mutually off-set each other's effects, and the cells are still able to proliferate (pART3 lacks any *cdc25* gene).

Figures 6A and 6B (*cdc25A*), 7A and 7B (*cdc25B*), and 8A and 8B (*cdc25C*) demonstrate the effect of expressing a human *cdc25* in a yeast "wee" background. Each of Figures 6A, 7A and 8A show that at the permissive temperature of 25°C (*wee1* is expressed) the cells are able to proliferate. However, as illustrated by Figures 6B, 7B and 8B, shifting the temperature to the non-permissive temperature of 37°C results in mitotic catastrophe. Microscopic analysis of the yeast cells present on the 37°C plates revealed that the expression of a human *cdc25* in a yeast *wee* background resulted in mitotic catastrophe for the cells.

Example 5

To provide a more stable transformant and uniform expression of the human *cdc25* gene, each of the resulting *ura4-cdc25* fragments of Examples 1-3 was used to transform a *ura4+* *S. pombe* strain. As in Example 4, each of the *S. pombe* strain carried a thermosensitive allele of its own *cdc25* gene, such as the *cdc25-22* phenotype, so that at non-

permissive temperatures the exogenous *cdc25* is principally responsible for activation of *cdc2*. In one embodiment, the *S. Pombe weel-50 cdc25-22 ura4+* strain was transformed with a *ura4-cdc25* fragment of Examples 1-3. This particular strain is generally viable at 25°C as well as the restrictive temperature of 37°C as the loss of endogenous *cdc25* activity is recovered by the concomitant loss of *weel* function at 37°C. However, integration and over expression of the human *cdc25*, as demonstrated in Example 4, can result in a mitotic catastrophic phenotype at 37°C as the *weel* checkpoint is impaired.

Example 6

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To assay the anti-mitotic activity of various candidate agents, the cells of Example 4 or 5 are either plated on a solid medium such as EMM plates or suspended in an appropriate vegetative broth such as YE.

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In the instance of plating on a solid medium, candidate agents are subsequently blotted onto the plate, and the plate incubated at the non-permissive temperature of 37°C. A halo of cell growth will form surrounding those agents able to at least partially inhibit a mitotic activator which can rescue the otherwise catastrophic cell.

20

Where growth of the cells is carried out in a vegetative broth, aliquots of cell/media are placed in the wells of microtitre plates and serial dilutions of candidate agents are added to the wells. The plates are incubated at 37°C, and the A_{540} for each well measured over time and compared to similar wells of cells/media which lack the candidate agent (e.g. negative controls). An increase in absorbance over time relative to the negative controls indicates positive proliferation of the cells and suggests an ability of a particular candidate agent to inhibit a mitotic activator.

25

All of the above-cited references and publications are hereby incorporated by reference.

30

Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, numerous equivalents to the specific assay and reagents described herein. Such equivalents are considered to be within the scope of this invention and are covered by the following claims.

35

SEQUENCE LISTING

(1) GENERAL INFORMATION:

5

(i) APPLICANT:

- (A) NAME: Mitotix, Inc.
- (B) STREET: One Kendall Square, Building 600
- (C) CITY: Cambridge
- (D) STATE: MA
- (E) COUNTRY: USA
- (F) POSTAL CODE (ZIP): 02139
- (G) TELEPHONE: (617) 225-0001
- (H) TELEFAX: (617) 225-0005

10

15

(ii) TITLE OF INVENTION: Assay and Reagents for Identifying
Anti-proliferative Agents

(iii) NUMBER OF SEQUENCES: 6

20

(iv) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: ASCII (text)

25

(vi) PRIOR APPLICATION DATA:

- (A) APPLICATION NUMBER: US 08/073,383
- (B) FILING DATE: 04-JUN-1993

30

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

35

- (A) LENGTH: 2420 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

40

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

45

- (A) NAME/KEY: CDS
- (B) LOCATION: 460..2031

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

50	CGAAAGGCCG GCCTTGGCTG CGACAGCCTG GGTAAGAGGT GTAGGTCGGC TTGGTTTTCT	60
	GCTACCCGGA GCTGGGCAAG CGGGTGGGGA GAACAGCGAA GACAGCGTGA GCCTGGGCCG	120
	TTGCCTCGAG GCTCTCGCCC GGCTTCTCTT GCCGACCCGC CACGTTTGTT TGGATTTAAT	180
55	CTTACAGCTG GTTGCCGGCG CCCGCCGCC CGCTGGCCTC GCGGTGTGAG AGGGAAGCAC	240

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	CCGTGCCTGT GTCTCGTGGC TGGCGCCTGG AGGGTCCGCA CACCCGCGCG GCCGCGCCGC	300
	TTTGCCCGCG GCAGCCGCGT CCCTGAACCG CGGAGTCGTG TTTGTGTTTG ACCCGCGGGC	360
5	GCCGGTGGCG CGCGGCCGAG GCCGGTGTCTG GCGGGGCGGG GCGGTCGCGC GGGAGGCAGA	420
	GGAAGAGGGA GCGGGAGCTC TGCGAGGCCG GCGCCGCC ATG GAA CTG GGC CCG	474
10	Met Glu Leu Gly Pro 1 5	
	AGC CCC GCA CCG CGC CGC CTG CTC TTC GCC TGC AGC CCC CCT CCC GCG	522
	Ser Pro Ala Pro Arg Arg Leu Leu Phe Ala Cys Ser Pro Pro Pro Ala	
15	10 15 20	
	TCG CAG CCC GTC GTG AAG GCG CTA TTT GGC GCT TCA GCC GCC GGG GGA	570
	Ser Gln Pro Val Val Lys Ala Leu Phe Gly Ala Ser Ala Ala Gly Gly	
	25 30 35	
20	CTG TCG CCT GTC ACC AAC CTG ACC GTC ACT ATG GAC CAG CTG CAG GGT	618
	Leu Ser Pro Val Thr Asn Leu Thr Val Thr Met Asp Gln Leu Gln Gly	
	40 45 50	
25	CTG GGC AGT GAT TAT GAG CAA CCA CTG GAG GTG AAG AAC AAC AGT AAT	666
	Leu Gly Ser Asp Tyr Glu Gln Pro Leu Glu Val Lys Asn Asn Ser Asn	
	55 60 65	
30	CTG CAG ATA ATG GGC TCC TCC AGA TCA ACA GAT TCA GGT TTC TGT CTA	714
	Leu Gln Ile Met Gly Ser Ser Arg Ser Thr Asp Ser Gly Phe Cys Leu	
	70 75 80 85	
	GAT TCT CCT GGG CCA TTG GAC AGT AAA GAA AAC CTT GAA AAT CCT ATG	762
35	Asp Ser Pro Gly Pro Leu Asp Ser Lys Glu Asn Leu Glu Asn Pro Met	
	90 95 100	
	AGA AGA ATA CAT TCC CTA CCT CAA AAG CTG TTG GGA TGT AGT CCA GCT	810
	Arg Arg Ile His Ser Leu Pro Gln Lys Leu Leu Gly Cys Ser Pro Ala	
	105 110 115	
40	CTG AAG AGG AGC CAT TCT GAT TCT CTT GAC CAT GAC ATC TTT CAG CTC	858
	Leu Lys Arg Ser His Ser Asp Ser Leu Asp His Asp Ile Phe Gln Leu	
	120 125 130	
45	ATC GAC CCA GAT GAG AAC AAG GAA AAT GAA GCC TTT GAG TTT AAG AAG	906
	Ile Asp Pro Asp Glu Asn Lys Glu Asn Glu Ala Phe Glu Phe Lys Lys	
	135 140 145	
50	CCA GTA AGA CCT GTA TCT CGT GGC TGC CTG CAC TCT CAT GGA CTC CAG	954
	Pro Val Arg Pro Val Ser Arg Gly Cys Leu His Ser His Gly Leu Gln	
	150 155 160 165	
	GAG GGT AAA GAT CTC TTC ACA CAG AGG CAG AAC TCT GCC CAG CTC GGA	1002
55	Glu Gly Lys Asp Leu Phe Thr Gln Arg Gln Asn Ser Ala Gln Leu Gly	
	170 175 180	

	ATG CTT TCC TCA AAT GAA AGA GAT AGC AGT GAA CCA GGG AAT TTC ATT	1050
	Met Leu Ser Ser Asn Glu Arg Asp Ser Ser Glu Pro Gly Asn Phe Ile	
	185 190 195	
5	CCT CTT TTT ACA CCC CAG TCA CCT GTG ACA GCC ACT TTG TCT GAT GAG	1098
	Pro Leu Phe Thr Pro Gln Ser Pro Val Thr Ala Thr Leu Ser Asp Glu	
	200 205 210	
10	GAT GAT GGC TTC GTG GAC CTT CTC GAT GGA GAC AAT CTG AAG AAT GAG	1146
	Asp Asp Gly Phe Val Asp Leu Leu Asp Gly Asp Asn Leu Lys Asn Glu	
	215 220 225	
15	GAG GAG ACC CCC TCG TGC ATG GCA AGC CTC TGG ACA GCT CCT CTC GTC	1194
	Glu Glu Thr Pro Ser Cys Met Ala Ser Leu Trp Thr Ala Pro Leu Val	
	230 235 240 245	
20	ATG AGA ACT ACA AAC CTT GAC AAC CGA TGC AAG CTG TTT GAC TCC CCT	1242
	Met Arg Thr Thr Asn Leu Asp Asn Arg Cys Lys Leu Phe Asp Ser Pro	
	250 255 260	
25	TCC CTG TGT AGC TCC AGC ACT CGG TCA GTG TTG AAG AGA CCA GAA CGT	1290
	Ser Leu Cys Ser Ser Ser Thr Arg Ser Val Leu Lys Arg Pro Glu Arg	
	265 270 275	
30	TCT CAA GAG GAG TCT CCA CCT GGA AGT ACA AAG AGG AGG AAG AGC ATG	1338
	Ser Gln Glu Glu Ser Pro Pro Gly Ser Thr Lys Arg Arg Lys Ser Met	
	280 285 290	
35	TCT GGG GCC AGC CCC AAA GAG TCA ACT AAT CCA GAG AAG GCC CAT GAG	1386
	Ser Gly Ala Ser Pro Lys Glu Ser Thr Asn Pro Glu Lys Ala His Glu	
	295 300 305	
40	ACT CTT CAT CAG TCT TTA TCC CTG GCA TCT TCC CCC AAA GGA ACC ATT	1434
	Thr Leu His Gln Ser Leu Ser Leu Ala Ser Ser Pro Lys Gly Thr Ile	
	310 315 320 325	
45	GAG AAC ATT TTG GAC AAT GAC CCA AGG GAC CTT ATA GGA GAC TTC TCC	1482
	Glu Asn Ile Leu Asp Asn Asp Pro Arg Asp Leu Ile Gly Asp Phe Ser	
	330 335 340	
50	AAG GGT TAT CTC TTT CAT ACA GTT GCT GGG AAA CAT CAG GAT TTA AAA	1530
	Lys Gly Tyr Leu Phe His Thr Val Ala Gly Lys His Gln Asp Leu Lys	
	345 350 355	
55	TAC ATC TCT CCA GAA ATT ATG GCA TCT GTT TTG AAT GGC AAG TTT GCC	1578
	Tyr Ile Ser Pro Glu Ile Met Ala Ser Val Leu Asn Gly Lys Phe Ala	
	360 365 370	
60	AAC CTC ATT AAA GAG TTT GTT ATC ATC GAC TGT CGA TAC CCA TAT GAA	1626
	Asn Leu Ile Lys Glu Phe Val Ile Ile Asp Cys Arg Tyr Pro Tyr Glu	
	375 380 385	
65	TAC GAG GGA GGC CAC ATC AAG GGT GCA GTG AAC TTG CAC ATG GAA GAA	1674
	Tyr Glu Gly Gly His Ile Lys Gly Ala Val Asn Leu His Met Glu Glu	
	390 395 400 405	

	GAG GTT GAA GAC TTC TTA TTG AAG AAG CCC ATT GTA CCT ACT GAT GGC	1722
	Glu Val Glu Asp Phe Leu Leu Lys Lys Pro Ile Val Pro Thr Asp Gly	
	410 415 420	
5	AAG CGT GTC ATT GTT GTG TTT CAC TGC GAG TTT TCT TCT GAG AGA GGT	1770
	Lys Arg Val Ile Val Val Phe His Cys Glu Phe Ser Ser Glu Arg Gly	
	425 430 435	
10	CCC CGC ATG TGC CGG TAT GTG AGA GAG AGA GAT CGC CTG GGT AAT GAA	1818
	Pro Arg Met Cys Arg Tyr Val Arg Glu Arg Asp Arg Leu Gly Asn Glu	
	440 445 450	
15	TAC CCC AAA CTC CAC TAC CCT GAG CTG TAT GTC CTG AAG GGG GGA TAC	1866
	Tyr Pro Lys Leu His Tyr Pro Glu Leu Tyr Val Leu Lys Gly Gly Tyr	
	455 460 465	
20	AAG GAG TTC TTT ATG AAA TGC CAG TCT TAC TGT GAG CCC CCT AGC TAC	1914
	Lys Glu Phe Phe Met Lys Cys Gln Ser Tyr Cys Glu Pro Pro Ser Tyr	
	470 475 480 485	
25	CGG CCC ATG CAC CAC GAG GAC TTT AAA GAA GAC CTG AAG AAG TTC CGC	1962
	Arg Pro Met His His Glu Asp Phe Lys Glu Asp Leu Lys Lys Phe Arg	
	490 495 500	
30	ACC AAG AGC CGG ACC TGG GCA GGG GAG AAG AGC AAG AGG GAG ATC TAC	2010
	Thr Lys Ser Arg Thr Trp Ala Gly Glu Lys Ser Lys Arg Glu Ile Tyr	
	505 510 515	
	AGT CGT CTG AAG AAG CTC TGAGGGCGGC AGGACCAGCC AGCAGCAGCC	2058
	Ser Arg Leu Lys Lys Leu	
	520	
35	CAAGCTTCCC TCCATCCCC TTTACCCTCT TTCCTGCAGA GAAACTTAAG CAAAGGGGAC	2118
	AGCTGTGTGA CATTTGGAGA GGGGGCCTGG GACTTCCATG CCTTAAACCT ACCTCCCACA	2178
40	CTCCCAAGGT TGGAGACCCA GGCCATCTTG CTGGCTACGC CTCTTCTGTC CCTGTTAGAC	2238
	GTCCTCCGTC CATTACAGAA CTGTGCCACA ATGCAGTTCT GAGCACCGTG TCAAGCTGCT	2298
	CTGAGCCACA GTGGGATGAA CCAGCCGGGG CCTTATCGGG CTCCAGCATC TCATGAGGGG	2358
45	AGAGGAGACG GAGGGGACTA GAGAAGTTTA CACAGAAATG CTGCTGGCCA AATAGCAAAG	2418
	AG	2420

50 (2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 523 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

55

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```

5  Met Glu Leu Gly Pro Ser Pro Ala Pro Arg Arg Leu Leu Phe Ala Cys
   1           5           10           15

Ser Pro Pro Pro Ala Ser Gln Pro Val Val Lys Ala Leu Phe Gly Ala
   20           25           30

10 Ser Ala Ala Gly Gly Leu Ser Pro Val Thr Asn Leu Thr Val Thr Met
   35           40           45

15 Asp Gln Leu Gln Gly Leu Gly Ser Asp Tyr Glu Gln Pro Leu Glu Val
   50           55           60

Lys Asn Asn Ser Asn Leu Gln Ile Met Gly Ser Ser Arg Ser Thr Asp
   65           70           75           80

20 Ser Gly Phe Cys Leu Asp Ser Pro Gly Pro Leu Asp Ser Lys Glu Asn
   85           90           95

25 Leu Glu Asn Pro Met Arg Arg Ile His Ser Leu Pro Gln Lys Leu Leu
   100          105          110

Gly Cys Ser Pro Ala Leu Lys Arg Ser His Ser Asp Ser Leu Asp His
   115          120          125

30 Asp Ile Phe Gln Leu Ile Asp Pro Asp Glu Asn Lys Glu Asn Glu Ala
   130          135          140

Phe Glu Phe Lys Lys Pro Val Arg Pro Val Ser Arg Gly Cys Leu His
   145          150          155          160

35 Ser His Gly Leu Gln Glu Gly Lys Asp Leu Phe Thr Gln Arg Gln Asn
   165          170          175

Ser Ala Gln Leu Gly Met Leu Ser Ser Asn Glu Arg Asp Ser Ser Glu
   180          185          190

40 Pro Gly Asn Phe Ile Pro Leu Phe Thr Pro Gln Ser Pro Val Thr Ala
   195          200          205

45 Thr Leu Ser Asp Glu Asp Asp Gly Phe Val Asp Leu Leu Asp Gly Asp
   210          215          220

Asn Leu Lys Asn Glu Glu Glu Thr Pro Ser Cys Met Ala Ser Leu Trp
   225          230          235          240

50 Thr Ala Pro Leu Val Met Arg Thr Thr Asn Leu Asp Asn Arg Cys Lys
   245          250          255

Leu Phe Asp Ser Pro Ser Leu Cys Ser Ser Ser Thr Arg Ser Val Leu
   260          265          270

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Lys Arg Pro Glu Arg Ser Gln Glu Glu Ser Pro Pro Gly Ser Thr Lys
275 280 285

5 Arg Arg Lys Ser Met Ser Gly Ala Ser Pro Lys Glu Ser Thr Asn Pro
290 295 300

Glu Lys Ala His Glu Thr Leu His Gln Ser Leu Ser Leu Ala Ser Ser
305 310 315 320

10 Pro Lys Gly Thr Ile Glu Asn Ile Leu Asp Asn Asp Pro Arg Asp Leu
325 330 335

Ile Gly Asp Phe Ser Lys Gly Tyr Leu Phe His Thr Val Ala Gly Lys
340 345 350

15 His Gln Asp Leu Lys Tyr Ile Ser Pro Glu Ile Met Ala Ser Val Leu
355 360 365

20 Asn Gly Lys Phe Ala Asn Leu Ile Lys Glu Phe Val Ile Ile Asp Cys
370 375 380

Arg Tyr Pro Tyr Glu Tyr Glu Gly Gly His Ile Lys Gly Ala Val Asn
385 390 395 400

25 Leu His Met Glu Glu Glu Val Glu Asp Phe Leu Leu Lys Lys Pro Ile
405 410 415

Val Pro Thr Asp Gly Lys Arg Val Ile Val Val Phe His Cys Glu Phe
420 425 430

Ser Ser Glu Arg Gly Pro Arg Met Cys Arg Tyr Val Arg Glu Arg Asp
435 440 445

35 Arg Leu Gly Asn Glu Tyr Pro Lys Leu His Tyr Pro Glu Leu Tyr Val
450 455 460

Leu Lys Gly Gly Tyr Lys Glu Phe Phe Met Lys Cys Gln Ser Tyr Cys
465 470 475 480

40 Glu Pro Pro Ser Tyr Arg Pro Met His His Glu Asp Phe Lys Glu Asp
485 490 495

Leu Lys Lys Phe Arg Thr Lys Ser Arg Thr Trp Ala Gly Glu Lys Ser
500 505 510

Lys Arg Glu Ile Tyr Ser Arg Leu Lys Lys Leu
515 520

50

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

55

(A) LENGTH: 2886 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 73..1773

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

15	CTGCCCTGCG CCCC GCCCTC CAGCCAGCCT GCCAGCTGTG CCGGCGTTTG TTGGTCTGCC	60
	GGCCCCGCCG CG ATG GAG GTG CCC CAG CCG GAG CCC GCG CCA GGC TCG	108
	Met Glu Val Pro Gln Pro Glu Pro Ala Pro Gly Ser	
	1 5 10	
20	GCT CTC AGT CCA GCA GGC GTG TGC GGT GGC GCC CAG CGT CCG GGC CAC	156
	Ala Leu Ser Pro Ala Gly Val Cys Gly Gly Ala Gln Arg Pro Gly His	
	15 20 25	
25	CTC CCG GGC CTC CTG CTG GGA TCT CAT GGC CTC CTG GGG TCC CCG GTG	204
	Leu Pro Gly Leu Leu Leu Gly Ser His Gly Leu Leu Gly Ser Pro Val	
	30 35 40	
30	CGG GCG GCC GCT TCC TCG CCG GTC ACC ACC CTC ACC CAG ACC ATG CAC	252
	Arg Ala Ala Ala Ser Ser Pro Val Thr Thr Leu Thr Gln Thr Met His	
	45 50 55 60	
	GAC CTC GCC GGG CTC GGC AGC CGC AGC CGC CTG ACG CAC CTA TCC CTG	300
	Asp Leu Ala Gly Leu Gly Ser Arg Ser Arg Leu Thr His Leu Ser Leu	
	65 70 75	
35	TCT CGA CGG GCA TCC GAA TCC TCC CTG TCG TCT GAA TCC TCC GAA TCT	348
	Ser Arg Arg Ala Ser Glu Ser Ser Leu Ser Ser Glu Ser Ser Glu Ser	
	80 85 90	
40	TCT GAT GCA GCT CTC TGC ATG GAT TCC CCC AGC CCT CTG GAC CCC CAC	396
	Ser Asp Ala Ala Leu Cys Met Asp Ser Pro Ser Pro Leu Asp Pro His	
	95 100 105	
45	ATG GCG GAG CAG ACG TTT GAA CAG GCC ATC CAG GCA GCC AGC CGG ATC	444
	Met Ala Glu Gln Thr Phe Glu Gln Ala Ile Gln Ala Ala Ser Arg Ile	
	110 115 120	
	ATT CGA AAC GAG CAG TTT GCC ATC AGA CGC TTC CAG TCT ATG CCG GTG	492
	Ile Arg Asn Glu Gln Phe Ala Ile Arg Arg Phe Gln Ser Met Pro Val	
50	125 130 135 140	
	AGG CTG CTG GGC CAC AGC CCC GTG CTT CGG AAC ATC ACC AAC TCC CAG	540
	Arg Leu Leu Gly His Ser Pro Val Leu Arg Asn Ile Thr Asn Ser Gln	
	145 150 155	
55	GCG CCC GAC GGC CGG AGG AAG AGC GAG GCG GGC AGT GGA GCT GCC AGC	588

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	Ala	Pro	Asp	Gly	Arg	Arg	Lys	Ser	Glu	Ala	Gly	Ser	Gly	Ala	Ala	Ser	
				160					165					170			
5	AGC	TCT	GGG	GAA	GAC	AAG	GAG	AAT	GAT	GGA	TTT	GTC	TTC	AAG	ATG	CCA	636
	Ser	Ser	Gly	Glu	Asp	Lys	Glu	Asn	Asp	Gly	Phe	Val	Phe	Lys	Met	Pro	
			175				180						185				
10	TGG	AAC	CCC	ACA	CAT	CCC	AGC	TCC	ACC	CAT	GCT	CTG	GCA	GAG	TGG	GCC	684
	Trp	Asn	Pro	Thr	His	Pro	Ser	Ser	Thr	His	Ala	Leu	Ala	Glu	Trp	Ala	
			190				195					200					
15	AGC	CGC	AGG	GAA	GCC	TTT	GCC	CAG	AGA	CCC	AGC	TCG	GCC	CCC	GAC	CTG	732
	Ser	Arg	Arg	Glu	Ala	Phe	Ala	Gln	Arg	Pro	Ser	Ser	Ala	Pro	Asp	Leu	
	205					210				215					220		
20	ATG	TGT	CTC	AGT	CCT	GAC	CCG	AAG	ATG	GAA	TTG	GAG	GAG	CTC	AGC	CCC	780
	Met	Cys	Leu	Ser	Pro	Asp	Pro	Lys	Met	Glu	Leu	Glu	Glu	Leu	Ser	Pro	
					225				230					235			
	CTG	GCC	CTA	GGT	CGC	TTC	TCT	CTG	ACC	CCT	GCA	GAG	GGG	GAT	ACT	GAG	828
	Leu	Ala	Leu	Gly	Arg	Phe	Ser	Leu	Thr	Pro	Ala	Glu	Gly	Asp	Thr	Glu	
				240				245					250				
25	GAA	GAT	GAT	GGA	TTT	GTG	GAC	ATC	CTA	GAG	AGT	GAC	TTA	AAG	GAT	GAT	876
	Glu	Asp	Asp	Gly	Phe	Val	Asp	Ile	Leu	Glu	Ser	Asp	Leu	Lys	Asp	Asp	
			255				260					265					
30	GAT	GCA	GTT	CCC	CCA	GGC	ATG	GAG	AGT	CTC	ATT	AGT	GCC	CCA	CTG	GTC	924
	Asp	Ala	Val	Pro	Pro	Gly	Met	Glu	Ser	Leu	Ile	Ser	Ala	Pro	Leu	Val	
		270				275					280						
35	AAG	ACC	TTG	GAA	AAG	GAA	GAG	GAA	AAG	GAC	CTC	GTC	ATG	TAC	AGC	AAG	972
	Lys	Thr	Leu	Glu	Lys	Glu	Glu	Glu	Lys	Asp	Leu	Val	Met	Tyr	Ser	Lys	
	285				290				295					300			
40	TGC	CAG	CGG	CTC	TTC	CGC	TCT	CCG	TCC	ATG	CCC	TGC	AGC	GTG	ATC	CGG	1020
	Cys	Gln	Arg	Leu	Phe	Arg	Ser	Pro	Ser	Met	Pro	Cys	Ser	Val	Ile	Arg	
				305				310						315			
	CCC	ATC	CTC	AAG	AGG	CTG	GAG	CGG	CCC	CAG	GAC	AGG	GAC	ACG	CCC	GTG	1068
	Pro	Ile	Leu	Lys	Arg	Leu	Glu	Arg	Pro	Gln	Asp	Arg	Asp	Thr	Pro	Val	
				320				325					330				
45	CAG	AAT	AAG	CGG	AGG	CGG	AGC	GTG	ACC	CCT	CCT	GAG	GAG	CAG	CAG	GAG	1116
	Gln	Asn	Lys	Arg	Arg	Arg	Ser	Val	Thr	Pro	Pro	Glu	Glu	Gln	Gln	Glu	
			335				340					345					
50	GCT	GAG	GAA	CCT	AAA	GCC	CGC	GCT	CTC	CGC	TCA	AAA	TCA	CTG	TGT	CAC	1164
	Ala	Glu	Glu	Pro	Lys	Ala	Arg	Ala	Leu	Arg	Ser	Lys	Ser	Leu	Cys	His	
		350				355					360						
55	GAT	GAG	ATC	GAG	AAC	CTC	CTG	GAC	AGT	GAC	CAC	CGA	GAG	CTG	ATT	GGA	1212
	Asp	Glu	Ile	Glu	Asn	Leu	Leu	Asp	Ser	Asp	His	Arg	Glu	Leu	Ile	Gly	
	365				370				375						380		

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	GAT TAC TCT AAG GCC TTC CTC CTA CAG ACA GTA GAC GGA AAG CAC CAA	1260
	Asp Tyr Ser Lys Ala Phe Leu Leu Gln Thr Val Asp Gly Lys His Gln	
	385 390 395	
5	GAC CTC AAG TAC ATC TCA CCA GAA ACG ATG GTG GCC CTA TTG ACG GGC	1308
	Asp Leu Lys Tyr Ile Ser Pro Glu Thr Met Val Ala Leu Leu Thr Gly	
	400 405 410	
10	AAG TTC AGC AAC ATC GTG GAT AAG TTT GTG ATT GTA GAC TGC AGA TAC	1356
	Lys Phe Ser Asn Ile Val Asp Lys Phe Val Ile Val Asp Cys Arg Tyr	
	415 420 425	
15	CCC TAT GAA TAT GAA GGC GGG CAC ATC AAG ACT GCG GTG AAC TTG CCC	1404
	Pro Tyr Glu Tyr Glu Gly Gly His Ile Lys Thr Ala Val Asn Leu Pro	
	430 435 440	
20	CTG GAA CGC GAC GCC GAG AGC TTC CTA CTG AAG AGC CCC ATC GCG CCC	1452
	Leu Glu Arg Asp Ala Glu Ser Phe Leu Leu Lys Ser Pro Ile Ala Pro	
	445 450 455 460	
25	TGT AGC CTG GAC AAG AGA GTC ATC CTC ATT TTC CAC TGT GAA TTC TCA	1500
	Cys Ser Leu Asp Lys Arg Val Ile Leu Ile Phe His Cys Glu Phe Ser	
	465 470 475	
30	TCT GAG CGT GGG CCC CGC ATG TGC CGT TTC ATC AGG GAA CGA GAC CGT	1548
	Ser Glu Arg Gly Pro Arg Met Cys Arg Phe Ile Arg Glu Arg Asp Arg	
	480 485 490	
35	GCT GTC AAC GAC TAC CCC AGC CTC TAC TAC CCT GAG ATG TAT ATC CTG	1596
	Ala Val Asn Asp Tyr Pro Ser Leu Tyr Tyr Pro Glu Met Tyr Ile Leu	
	495 500 505	
40	AAA GGC GGC TAC AAG GAG TTC TTC CCT CAG CAC CCG AAC TTC TGT GAA	1644
	Lys Gly Gly Tyr Lys Glu Phe Phe Pro Gln His Pro Asn Phe Cys Glu	
	510 515 520	
45	CCC CAG GAC TAC CGG CCC ATG AAC CAC GAG GCC TTC AAG GAT GAG CTA	1692
	Pro Gln Asp Tyr Arg Pro Met Asn His Glu Ala Phe Lys Asp Glu Leu	
	525 530 535 540	
50	AAG ACC TTC CGC CTC AAG ACT CGC AGC TGG GCT GGG GAG CGG AGC CGG	1740
	Lys Thr Phe Arg Leu Lys Thr Arg Ser Trp Ala Gly Glu Arg Ser Arg	
	545 550 555	
55	CGG GAG CTC TGT AGC CGG CTG CAG GAC CAG TGAGGGGCCT GCGCCAGTCC	1790
	Arg Glu Leu Cys Ser Arg Leu Gln Asp Gln	
	560 565	
55	TGCTACCTCC CTTGCCTTTC GAGGCCTGAA GCCAGCTGCC CTATGGGCCT GCCGGGCTGA	1850
	GGGCCTGCTG GAGGCCTCAG GTGCTGTCCA TGGGAAAGAT GGTGTGGTGT CCTGCCTGTC	1910
	TGCCCCAGCC CAGATTCCCC TGTGTCATCC CATCATTTTC CATATCCTGG TGCCCCCACC	1970
55	CCCTGGAAGA GCCCAGTCTG TTGAGTTAGT TAAGTTGGGT TAATACCAGC TTAAAGTCAG	2030

TATTTTGTGT CCTCCAGGAG CTTCTTGTTT CTTGTTAGG GTTAACCCTT CATCTTCCTG 2090
 TGTCTTGAAA CGCTCCAGAG CTAAACTCCT TCCTGGCCTG AGAGTCAGCT CTCTGCCCTG 2150
 5 TGTACTTCCC GGGCCAGGGC TGCCCCTAAT CTCTGTAGGA ACCGTGGTAT GTCTGCCATG 2210
 TTGCCCCCTT CTCTTTTCCC CTTTCCTGTC CCACCATACG AGCACCTCCA GCCTGAACAG 2270
 AAGCTCTTAC TCTTTCTTAT TTCAGTGTTA CCTGTGTGCT TGGTCTGTTT GACTTTACGC 2330
 10 CCATCTCAGG ACACTTCCGT AGACTGTTTA GGTTCCTCTG TCAAATATCA GTTACCCACT 2390
 CGGTCCCAGT TTTGTTGCCC CAGAAAGGGA TGTATTATC CTTGGGGGCT CCCAGGGCAA 2450
 15 GGGTTAAGGC CTGAATCATG AGCCTGCTGG AAGCCCAGCC CCTACTGCTG TGAACCCTGG 2510
 GGCCTGACTG CTCAGAACTT GCTGCTGTCT TGTGCGGAT GGATGGAAGG TTGGATGGAT 2570
 GGGTGGATGG CCGTGGATGG CCGTGGATGC GCAGTGCCTT GCATACCCAA ACCAGGTGGG 2630
 20 AGCGTTTTGT TGAGCATGAC ACCTGCAGCA GGAATATATG TGTGCCTATT TGTGTGGACA 2690
 AAAATATTTA CACTTAGGGT TTGGAGCTAT TCAAGAAGAA ATGTCACAGA AGCAGCTAAA 2750
 25 CCAAGGACTG AGCACCTCTT GGATTCTGAA TCTCAATATG GGGGCAGGGC TGTGCTTGAA 2810
 GGCCCTGCTG AGTCATCTGT TAGGGCCTTG GTTCAATAAA GCACTGAGCA AGTTGAGAAA 2870
 AAAAAAAAAA AAAAAA 2886
 30

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
 35 (A) LENGTH: 566 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein
 40

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Met Glu Val Pro Gln Pro Glu Pro Ala Pro Gly Ser Ala Leu Ser Pro
 1 5 10 15
 45 Ala Gly Val Cys Gly Gly Ala Gln Arg Pro Gly His Leu Pro Gly Leu
 20 25 30
 Leu Leu Gly Ser His Gly Leu Leu Gly Ser Pro Val Arg Ala Ala Ala
 35 40 45
 Ser Ser Pro Val Thr Thr Leu Thr Gln Thr Met His Asp Leu Ala Gly
 50 55 60
 55 Leu Gly Ser Arg Ser Arg Leu Thr His Leu Ser Leu Ser Arg Arg Ala

	65	70	75	80
	Ser Glu Ser Ser Leu Ser Ser Glu Ser Ser Glu Ser Ser Asp Ala Ala	85	90	95
5	Leu Cys Met Asp Ser Pro Ser Pro Leu Asp Pro His Met Ala Glu Gln	100	105	110
10	Thr Phe Glu Gln Ala Ile Gln Ala Ala Ser Arg Ile Ile Arg Asn Glu	115	120	125
	Gln Phe Ala Ile Arg Arg Phe Gln Ser Met Pro Val Arg Leu Leu Gly	130	135	140
15	His Ser Pro Val Leu Arg Asn Ile Thr Asn Ser Gln Ala Pro Asp Gly	145	150	155
	Arg Arg Lys Ser Glu Ala Gly Ser Gly Ala Ala Ser Ser Ser Gly Glu	165	170	175
20	Asp Lys Glu Asn Asp Gly Phe Val Phe Lys Met Pro Trp Asn Pro Thr	180	185	190
25	His Pro Ser Ser Thr His Ala Leu Ala Glu Trp Ala Ser Arg Arg Glu	195	200	205
	Ala Phe Ala Gln Arg Pro Ser Ser Ala Pro Asp Leu Met Cys Leu Ser	210	215	220
30	Pro Asp Pro Lys Met Glu Leu Glu Glu Leu Ser Pro Leu Ala Leu Gly	225	230	235
	Arg Phe Ser Leu Thr Pro Ala Glu Gly Asp Thr Glu Glu Asp Asp Gly	245	250	255
35	Phe Val Asp Ile Leu Glu Ser Asp Leu Lys Asp Asp Asp Ala Val Pro	260	265	270
40	Pro Gly Met Glu Ser Leu Ile Ser Ala Pro Leu Val Lys Thr Leu Glu	275	280	285
	Lys Glu Glu Glu Lys Asp Leu Val Met Tyr Ser Lys Cys Gln Arg Leu	290	295	300
45	Phe Arg Ser Pro Ser Met Pro Cys Ser Val Ile Arg Pro Ile Leu Lys	305	310	315
	Arg Leu Glu Arg Pro Gln Asp Arg Asp Thr Pro Val Gln Asn Lys Arg	325	330	335
50	Arg Arg Ser Val Thr Pro Pro Glu Glu Gln Gln Glu Ala Glu Glu Pro	340	345	350
55	Lys Ala Arg Ala Leu Arg Ser Lys Ser Leu Cys His Asp Glu Ile Glu	355	360	365

Asn Leu Leu Asp Ser Asp His Arg Glu Leu Ile Gly Asp Tyr Ser Lys
 370 375 380

5
 Ala Phe Leu Leu Gln Thr Val Asp Gly Lys His Gln Asp Leu Lys Tyr
 385 390 395 400

10
 Ile Ser Pro Glu Thr Met Val Ala Leu Leu Thr Gly Lys Phe Ser Asn
 405 410 415

Ile Val Asp Lys Phe Val Ile Val Asp Cys Arg Tyr Pro Tyr Glu Tyr
 420 425 430

15
 Glu Gly Gly His Ile Lys Thr Ala Val Asn Leu Pro Leu Glu Arg Asp
 435 440 445

Ala Glu Ser Phe Leu Leu Lys Ser Pro Ile Ala Pro Cys Ser Leu Asp
 450 455 460

20
 Lys Arg Val Ile Leu Ile Phe His Cys Glu Phe Ser Ser Glu Arg Gly
 465 470 475 480

25
 Pro Arg Met Cys Arg Phe Ile Arg Glu Arg Asp Arg Ala Val Asn Asp
 485 490 495

Tyr Pro Ser Leu Tyr Tyr Pro Glu Met Tyr Ile Leu Lys Gly Gly Tyr
 500 505 510

30
 Lys Glu Phe Phe Pro Gln His Pro Asn Phe Cys Glu Pro Gln Asp Tyr
 515 520 525

Arg Pro Met Asn His Glu Ala Phe Lys Asp Glu Leu Lys Thr Phe Arg
 530 535 540

35
 Leu Lys Thr Arg Ser Trp Ala Gly Glu Arg Ser Arg Arg Glu Leu Cys
 545 550 555 560

40
 Ser Arg Leu Gln Asp Gln
 565

45 (2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- 50 (A) LENGTH: 2062 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

55

(ix) FEATURE:

(A) NAME/KEY: CDS
(B) LOCATION: 211..1631

5 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

	CAGGAAGACT CTGAGTCCGA CGTTGGCCTA CCCAGTCGGA AGGCAGAGCT GCAATCTAGT	60
	TAACCTACCTC CTTTCCCCTA GATTTCCCTT CATTCTGCTC AAGTCTTCGC CTGTGTCCGA	120
10	TCCCTATCTA CTTTCTCTCC TCTTGTAGCA AGCCTCAGAC TCCAGGCTTG AGCTAGGTTT	180
	TGTTTTTCTC CTGGTGAGAA TTCGAAGACC ATG TCT ACG GAA CTC TTC TCA TCC	234
	Met Ser Thr Glu Leu Phe Ser Ser	
15	1 5	
	ACA AGA GAG GAA GGA AGC TCT GGC TCA GGA CCC AGT TTT AGG TCT AAT	282
	Thr Arg Glu Glu Gly Ser Ser Gly Ser Gly Pro Ser Phe Arg Ser Asn	
	10 15 20	
20	CAA AGG AAA ATG TTA AAC CTG CTC CTG GAG AGA GAC ACT TCC TTT ACC	330
	Gln Arg Lys Met Leu Asn Leu Leu Leu Glu Arg Asp Thr Ser Phe Thr	
	25 30 35 40	
25	GTC TGT CCA GAT GTC CCT AGA ACT CCA GTG GGC AAA TTT CTT GGT GAT	378
	Val Cys Pro Asp Val Pro Arg Thr Pro Val Gly Lys Phe Leu Gly Asp	
	45 50 55	
	TCT GCA AAC CTA AGC ATT TTG TCT GGA GGA ACC CCA AAA TGT TGC CTC	426
30	Ser Ala Asn Leu Ser Ile Leu Ser Gly Gly Thr Pro Lys Cys Cys Leu	
	60 65 70	
	GAT CTT TCG AAT CTT AGC AGT GGG GAG ATA ACT GCC ACT CAG CTT ACC	474
	Asp Leu Ser Asn Leu Ser Ser Gly Glu Ile Thr Ala Thr Gln Leu Thr	
35	75 80 85	
	ACT TCT GCA GAC CTT GAT GAA ACT GGT CAC CTG GAT TCT TCA GGA CTT	522
	Thr Ser Ala Asp Leu Asp Glu Thr Gly His Leu Asp Ser Ser Gly Leu	
	90 95 100	
40	CAG GAA GTG CAT TTA GCT GGG ATG AAT CAT GAC CAG CAC CTA ATG AAA	570
	Gln Glu Val His Leu Ala Gly Met Asn His Asp Gln His Leu Met Lys	
	105 110 115 120	
45	TGT AGC CCA GCA CAG CTT CTT TGT AGC ACT CCG AAT GGT TTG GAC CGT	618
	Cys Ser Pro Ala Gln Leu Leu Cys Ser Thr Pro Asn Gly Leu Asp Arg	
	125 130 135	
	GGC CAT AGA AAG AGA GAT GCA ATG TGT AGT TCA TCT GCA AAT AAA GAA	666
50	Gly His Arg Lys Arg Asp Ala Met Cys Ser Ser Ser Ala Asn Lys Glu	
	140 145 150	
	AAT GAC AAT GGA AAC TTG GTG GAC AGT GAA ATG AAA TAT TTG GGC AGT	714
	Asn Asp Asn Gly Asn Leu Val Asp Ser Glu Met Lys Tyr Leu Gly Ser	
55	155 160 165	

	CCC	ATT	ACT	ACT	GTT	CCA	AAA	TTG	GAT	AAA	AAT	CCA	AAC	CTA	GGA	GAA	762
	Pro	Ile	Thr	Thr	Val	Pro	Lys	Leu	Asp	Lys	Asn	Pro	Asn	Leu	Gly	Glu	
	170						175					180					
5	GAC	CAG	GCA	GAA	GAG	ATT	TCA	GAT	GAA	TTA	ATG	GAG	TTT	TCC	CTG	AAA	810
	Asp	Gln	Ala	Glu	Glu	Ile	Ser	Asp	Glu	Leu	Met	Glu	Phe	Ser	Leu	Lys	
	185					190				195					200		
10	GAT	CAA	GAA	GCA	AAG	GTG	AGC	AGA	AGT	GGC	CTA	TAT	CGC	TCC	CCG	TCG	858
	Asp	Gln	Glu	Ala	Lys	Val	Ser	Arg	Ser	Gly	Leu	Tyr	Arg	Ser	Pro	Ser	
					205					210					215		
15	ATG	CCA	GAG	AAC	TTG	AAC	AGG	CCA	AGA	CTG	AAG	CAG	GTG	GAA	AAA	TTC	906
	Met	Pro	Glu	Asn	Leu	Asn	Arg	Pro	Arg	Leu	Lys	Gln	Val	Glu	Lys	Phe	
				220				225						230			
20	AAG	GAC	AAC	ACA	ATA	CCA	GAT	AAA	GTT	AAA	AAA	AAG	TAT	TTT	TCT	GGC	954
	Lys	Asp	Asn	Thr	Ile	Pro	Asp	Lys	Val	Lys	Lys	Lys	Tyr	Phe	Ser	Gly	
			235					240						245			
25	CAA	GGA	AAG	CTC	AGG	AAG	GGC	TTA	TGT	TTA	AAG	AAG	ACA	GTC	TCT	CTG	1002
	Gln	Gly	Lys	Leu	Arg	Lys	Gly	Leu	Cys	Leu	Lys	Lys	Thr	Val	Ser	Leu	
		250					255					260					
30	TGT	GAC	ATT	ACT	ATC	ACT	CAG	ATG	CTG	GAG	GAA	GAT	TCT	AAC	CAG	GGG	1050
	Cys	Asp	Ile	Thr	Ile	Thr	Gln	Met	Leu	Glu	Glu	Asp	Ser	Asn	Gln	Gly	
	265					270					275					280	
35	CAC	CTG	ATT	GGT	GAT	TTT	TCC	AAG	GTA	TGT	GCG	CTG	CCA	ACC	GTG	TCA	1098
	His	Leu	Ile	Gly	Asp	Phe	Ser	Lys	Val	Cys	Ala	Leu	Pro	Thr	Val	Ser	
				285						290					295		
40	GGG	AAA	CAC	CAA	GAT	CTG	AAG	TAT	GTC	AAC	CCA	GAA	ACA	GTG	GCT	GCC	1146
	Gly	Lys	His	Gln	Asp	Leu	Lys	Tyr	Val	Asn	Pro	Glu	Thr	Val	Ala	Ala	
				300					305					310			
45	TTA	CTG	TCG	GGG	AAG	TTC	CAG	GGT	CTG	ATT	GAG	AAG	TTT	TAT	GTC	ATT	1194
	Leu	Leu	Ser	Gly	Lys	Phe	Gln	Gly	Leu	Ile	Glu	Lys	Phe	Tyr	Val	Ile	
			315					320						325			
50	GAT	TGT	CGC	TAT	CCA	TAT	GAG	TAT	CTG	GGA	GGA	CAC	ATC	CAG	GGA	GCC	1242
	Asp	Cys	Arg	Tyr	Pro	Tyr	Glu	Tyr	Leu	Gly	Gly	His	Ile	Gln	Gly	Ala	
		330						335					340				
55	TTA	AAC	TTA	TAT	AGT	CAG	GAA	GAA	CTG	TTT	AAC	TTC	TTT	CTG	AAG	AAG	1290
	Leu	Asn	Leu	Tyr	Ser	Gln	Glu	Glu	Leu	Phe	Asn	Phe	Phe	Leu	Lys	Lys	
		345				350					355				360		
60	CCC	ATC	GTC	CCT	TTG	GAC	ACC	CAG	AAG	AGA	ATA	ATC	ATC	GTG	TTC	CAC	1338
	Pro	Ile	Val	Pro	Leu	Asp	Thr	Gln	Lys	Arg	Ile	Ile	Ile	Val	Phe	His	
					365					370					375		
65	TGT	GAA	TTC	TCC	TCA	GAG	AGG	GGC	CCC	CGA	ATG	TGC	CGC	TGT	CTG	CGT	1386
	Cys	Glu	Phe	Ser	Ser	Glu	Arg	Gly	Pro	Arg	Met	Cys	Arg	Cys	Leu	Arg	
			380						385					390			

GAA GAG GAC AGG TCT CTG AAC CAG TAT CCT GCA TTG TAC TAC CCA GAG 1434
 Glu Glu Asp Arg Ser Leu Asn Gln Tyr Pro Ala Leu Tyr Tyr Pro Glu
 395 400 405

5 CTA TAT ATC CTT AAA GGC GGC TAC AGA GAC TTC TTT CCA GAA TAT ATG 1482
 Leu Tyr Ile Leu Lys Gly Gly Tyr Arg Asp Phe Phe Pro Glu Tyr Met
 410 415 420

10 GAA CTG TGT GAA CCA CAG AGC TAC TGC CCT ATG CAT CAT CAG GAC CAC 1530
 Glu Leu Cys Glu Pro Gln Ser Tyr Cys Pro Met His His Gln Asp His
 425 430 435 440

15 AAG ACT GAG TTG CTG AGG TGT CGA AGC CAG AGC AAA GTG CAG GAA GGG 1578
 Lys Thr Glu Leu Leu Arg Cys Arg Ser Gln Ser Lys Val Gln Glu Gly
 445 450 455

20 GAG CGG CAG CTG CGG GAG CAG ATT GCC CTT CTG GTG AAG GAC ATG AGC 1626
 Glu Arg Gln Leu Arg Glu Gln Ile Ala Leu Leu Val Lys Asp Met Ser
 460 465 470

CCA TG ATAACATTCC AGCCACTGGC TGCTAACAAG TCACCAAAAA GACACTGCAG 1681
 Pro

25 AAACCCTGAG CAGAAAGAGG CCTTCTGGAT GGCCAAACCC AAGATTATTA AAAGATGTCT 1741

CTGCAAACCA ACAGGCTACC AACTTGTATC CAGGCCTGGG AATGGATTAG GTTTCAGCAG 1801

30 AGCTGAAAGC TGGTGGCCAG AGTCCTGGAG CTGGCTCTAT AAGGCAGCCT TGAGTGCATA 1861

GAGATTTGTA TTGGTTCAGG GAACTCTGGC ATTCCTTTTC CCAACTCCTC ATGTCTTCTC 1921

35 ACAAGCCAGC CAACTCTTTC TCTCTGGGCT TCGGGCTATG CAAGAGCGTT GTCTACCTTC 1981

TTTCTTTGTA TTTTCCTTCT TTGTTTCCCC CTCTTTCTTT TTTAAAAATG GAAAAATAAA 2041

CACTACAGAA TGAGAAAAAA A 2062

40 (2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

45 (A) LENGTH: 473 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

50 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Met Ser Thr Glu Leu Phe Ser Ser Thr Arg Glu Glu Gly Ser Ser Gly
 1 5 10 15

55 Ser Gly Pro Ser Phe Arg Ser Asn Gln Arg Lys Met Leu Asn Leu Leu
 20 25 30

	Leu	Glu	Arg	Asp	Thr	Ser	Phe	Thr	Val	Cys	Pro	Asp	Val	Pro	Arg	Thr	
			35					40					45				
5	Pro	Val	Gly	Lys	Phe	Leu	Gly	Asp	Ser	Ala	Asn	Leu	Ser	Ile	Leu	Ser	
		50					55					60					
	Gly	Gly	Thr	Pro	Lys	Cys	Cys	Leu	Asp	Leu	Ser	Asn	Leu	Ser	Ser	Gly	
	65					70					75					80	
10	Glu	Ile	Thr	Ala	Thr	Gln	Leu	Thr	Thr	Ser	Ala	Asp	Leu	Asp	Glu	Thr	
					85					90					95		
	Gly	His	Leu	Asp	Ser	Ser	Gly	Leu	Gln	Glu	Val	His	Leu	Ala	Gly	Met	
15				100					105					110			
	Asn	His	Asp	Gln	His	Leu	Met	Lys	Cys	Ser	Pro	Ala	Gln	Leu	Leu	Cys	
			115					120					125				
20	Ser	Thr	Pro	Asn	Gly	Leu	Asp	Arg	Gly	His	Arg	Lys	Arg	Asp	Ala	Met	
		130					135						140				
	Cys	Ser	Ser	Ser	Ala	Asn	Lys	Glu	Asn	Asp	Asn	Gly	Asn	Leu	Val	Asp	
	145						150				155					160	
25	Ser	Glu	Met	Lys	Tyr	Leu	Gly	Ser	Pro	Ile	Thr	Thr	Val	Pro	Lys	Leu	
					165					170					175		
	Asp	Lys	Asn	Pro	Asn	Leu	Gly	Glu	Asp	Gln	Ala	Glu	Glu	Ile	Ser	Asp	
30				180					185					190			
	Glu	Leu	Met	Glu	Phe	Ser	Leu	Lys	Asp	Gln	Glu	Ala	Lys	Val	Ser	Arg	
		195						200					205				
35	Ser	Gly	Leu	Tyr	Arg	Ser	Pro	Ser	Met	Pro	Glu	Asn	Leu	Asn	Arg	Pro	
		210					215					220					
	Arg	Leu	Lys	Gln	Val	Glu	Lys	Phe	Lys	Asp	Asn	Thr	Ile	Pro	Asp	Lys	
	225						230				235					240	
40	Val	Lys	Lys	Lys	Tyr	Phe	Ser	Gly	Gln	Gly	Lys	Leu	Arg	Lys	Gly	Leu	
					245					250					255		
	Cys	Leu	Lys	Lys	Thr	Val	Ser	Leu	Cys	Asp	Ile	Thr	Ile	Thr	Gln	Met	
45				260					265					270			
	Leu	Glu	Glu	Asp	Ser	Asn	Gln	Gly	His	Leu	Ile	Gly	Asp	Phe	Ser	Lys	
		275						280					285				
50	Val	Cys	Ala	Leu	Pro	Thr	Val	Ser	Gly	Lys	His	Gln	Asp	Leu	Lys	Tyr	
		290					295					300					
	Val	Asn	Pro	Glu	Thr	Val	Ala	Ala	Leu	Leu	Ser	Gly	Lys	Phe	Gln	Gly	
	305					310					315					320	
55	Leu	Ile	Glu	Lys	Phe	Tyr	Val	Ile	Asp	Cys	Arg	Tyr	Pro	Tyr	Glu	Tyr	

325 330 335

Leu Gly Gly His Ile Gln Gly Ala Leu Asn Leu Tyr Ser Gln Glu Glu
340 345 350

5
Leu Phe Asn Phe Phe Leu Lys Lys Pro Ile Val Pro Leu Asp Thr Gln
355 360 365

10
Lys Arg Ile Ile Ile Val Phe His Cys Glu Phe Ser Ser Glu Arg Gly
370 375 380

Pro Arg Met Cys Arg Cys Leu Arg Glu Glu Asp Arg Ser Leu Asn Gln
385 390 395 400

15
Tyr Pro Ala Leu Tyr Tyr Pro Glu Leu Tyr Ile Leu Lys Gly Gly Tyr
405 410 415

Arg Asp Phe Phe Pro Glu Tyr Met Glu Leu Cys Glu Pro Gln Ser Tyr
420 425 430

20
Cys Pro Met His His Gln Asp His Lys Thr Glu Leu Leu Arg Cys Arg
435 440 445

25
Ser Gln Ser Lys Val Gln Glu Gly Glu Arg Gln Leu Arg Glu Gln Ile
450 455 460

Ala Leu Leu Val Lys Asp Met Ser Pro
465 470

30

What is claimed:

1. An assay for identifying an anti-proliferative agent, comprising
 - 5 i. providing a cell having an impaired cell-cycle checkpoint, wherein the impaired cell-cycle checkpoint inhibits proliferation of the cell by causing either premature progression of the cell through at least a portion of a cell-cycle or inhibition of normal progression of the cell through at least a portion of a cell-cycle;
 - 10 ii. contacting the cell with a candidate agent;
 - 15 iii. measuring a level of proliferation of the cell in the presence of the candidate agent; and
 - iv. comparing the level of proliferation of the cell in the presence of the candidate agent to a level of proliferation of the cell in the absence of the candidate agent, wherein an increase in the level of proliferation in the presence of the candidate agent is indicative of anti-proliferative activity of the candidate agent.
2. The assay of claim 1, wherein the cell-cycle is a mitotic cell-cycle.
- 20 3. The assay of claim 2, wherein the cell is a hyper-mitotic cell and the impaired cell-cycle checkpoint causes premature progression of the cell through at least a portion of the mitotic cell-cycle sufficient to cause the cell to conclude in mitotic catastrophe.
- 25 4. The assay of claim 2, wherein the cell is a hypo-mitotic cell and the impaired cell-cycle checkpoint causes inhibition of progression of the cell through at least a portion of the mitotic cell-cycle sufficient to inhibit mitosis.
5. The assay of claim 1, wherein the cell-cycle is a meiotic cell-cycle.
- 30 6. The assay of claim 5, wherein the cell is a hyper-meiotic cell and the impaired cell-cycle checkpoint causes premature progression of the cell through at least a portion of the meiotic cell-cycle sufficient to cause the cell to conclude in meiotic catastrophe.
- 35 7. The assay of claim 5, wherein the cell is a hypo-meiotic cell and the impaired cell-cycle checkpoint causes inhibition of progression of the cell through at least a portion of the meiotic cell-cycle sufficient to inhibit meiosis.

8. An assay for identifying an anti-mitotic agent, comprising
 - i. providing a cell having an impaired cell-cycle checkpoint which causes premature progression of the cell through at least a portion of a mitotic cell-cycle resulting in inhibition of proliferation of the cell;
 - ii. contacting the cell with a candidate agent;
 - iii. measuring a level of proliferation of the cell in the presence of the candidate agent; and
 - iv. comparing the level of proliferation of the cell in the presence of the candidate agent to a level of proliferation of the cell in the absence of the candidate agent, wherein an increase in the level of proliferation in the presence of the candidate agent is indicative of anti-mitotic activity of the candidate agent.
9. The assay of claim 8, wherein the cell-cycle checkpoint comprises a G1/S checkpoint.
10. The assay of claim 8, wherein the cell-cycle checkpoint comprises a G2/M checkpoint.
11. The assay of claim 8, wherein the cell-cycle checkpoint is conditionally impairable and the level of proliferation of the cell in the presence and the absence of the candidate agent is measured under conditions wherein the checkpoint is impaired.
12. An assay for identifying an inhibitor of a cdc25 phosphatase, comprising
 - i. providing a cell having an impaired checkpoint which can cause premature entry of the cell into mitosis resulting in inhibition of proliferation of the cell, the premature entry into mitosis being mediated at least in part by the cdc25 phosphatase;
 - ii. contacting the cell with a candidate agent;
 - iii. measuring a level of proliferation of the cell in the presence of the candidate agent; and
 - iv. comparing the level of proliferation of the cell in the presence of the candidate agent to a level of proliferation of the cell in the absence of the candidate agent, wherein an increase in the level of proliferation in the presence of the candidate agent is indicative of inhibition of the cdc25 phosphatase by the candidate agent.

13. The assay of claim 12, wherein the cell-cycle checkpoint impairment results in entry of the cell into mitosis before completion of replication or repair of genomic DNA of the cell.
14. The assay of claim 13, wherein the cell-cycle checkpoint impairment comprises a reduction of inhibitory phosphorylation of a cdk.
15. The assay of claim 14, wherein the cell-cycle checkpoint impairment comprises an impaired wee1 protein kinase activity, an impaired mik1 protein kinase activity, or an over-expression of a nim1 gene product.
16. The assay of claim 12, wherein the cell-cycle checkpoint impairment is induced by treatment of the cell with a hyper-mitotic agent.
17. The assay of claim 16, wherein the hyper-mitotic agent is selected from a group consisting of caffeine, 2-aminopurine, 6-dimethylaminopurine, and okadaic acid.
18. The assay of claim 12, wherein the cell-cycle checkpoint is conditionally impairable and the level of proliferation of the cell in the presence and the absence of the candidate agent is measured under conditions wherein the cell-cycle checkpoint is impaired.
19. The assay of claim 12, wherein the cell is a yeast cell.
20. The assay of claim 19, wherein the yeast cell is a species of the genus *Schizosaccharomyces*.
21. The assay of claim 12, wherein the cdc25 phosphatase is a recombinant gene product expressed in the cell.
22. The assay of claim 12, wherein the cdc25 phosphatase is a human cdc25 or homolog thereof.
23. The assay of claim 12, wherein the cdc25 phosphatase is a cdc25 or homolog thereof of a human pathogen.

24. The assay of claim 23, wherein the cdc25 phosphatase is derived from a human pathogen which is implicated in mycotic infection.
- 5 25. The assay of claim 24, wherein the mycotic infection is a mycosis selected from a group consisting of candidiasis, aspergillosis, mucormycosis, blastomycosis, geotrichosis, cryptococcosis, chromoblastomycosis, penicilliosis, conidiosporosis, nocardiosis, coccidioidomycosis, histoplasmosis, maduromycosis, rhinosporidiosis, monoliasis, para-actinomycosis, and sporotrichosis.
- 10 26. The assay of claim 24, wherein the human pathogen is selected from a group consisting of *Candida albicans*, *Candida stellatoidea*, *Candida tropicalis*, *Candida parapsilosis*, *Candida krusei*, *Candida pseudotropicalis*, *Candida quillermundii*, *Candida rugosa*, *Aspergillus fumigatus*, *Aspergillus flavus*, *Aspergillus niger*, *Aspergillus nidulans*, *Aspergillus terreus*, *Rhizopus arrhizus*, *Rhizopus oryzae*,
15 *Absidia corymbifera*, *Absidia ramosa*, and *Mucor pusillus*.
27. The assay of claim 23, wherein the human pathogen is a *Pneumocystis* or a *Toxoplasma*.
- 20 28. An assay for identifying an anti-mitotic agent, comprising
- i. providing a cell having an impaired cell-cycle checkpoint which inhibits progression of the cell through at least a portion of a mitotic cell-cycle resulting in inhibition of proliferation of the cell;
 - ii. contacting the cell with a candidate agent;

25 iii. measuring a level of proliferation of the cell in the presence of the candidate agent; and

 - iv. comparing the level of proliferation of the cell in the presence of the candidate agent to a level of proliferation of the cell in the absence of the candidate agent, wherein an increase in the level of proliferation in the
30 presence of the candidate agent is indicative of anti-mitotic activity of the candidate agent.
29. The assay of claim 28, wherein the cell-cycle checkpoint comprises a G1/S checkpoint.
- 35 30. The assay of claim 28, wherein the cell-cycle checkpoint comprises a G2/M checkpoint.

31. The assay of claim 28, wherein the cell-cycle checkpoint is conditionally impairable and the level of proliferation of the cell in the presence and the absence of the candidate agent is measured under conditions wherein the checkpoint is impaired.
- 5
32. An assay for identifying an inhibitor of a cdc25 phosphatase, comprising
- 10
- i. providing a *Schizosaccharomyces* cell having a conditionally impairable wee1 protein kinase which can cause inhibition of proliferation of the *Schizosaccharomyces* cell by facilitating premature entry of the *Schizosaccharomyces* cell into mitosis under conditions wherein the wee1 kinase is impaired, the premature entry into mitosis being mediated at least in part by the cdc25 phosphatase and a reduced level of inhibitory phosphorylation of a cdc2 protein kinase by the wee1 protein kinase;
- 15
- ii. contacting the *Schizosaccharomyces* cell with a test compound under the conditions wherein the wee1 kinase is impaired;
- iii. measuring a level of proliferation of the *Schizosaccharomyces* cell in the presence of the test compound; and
- 20
- iv. comparing the level of proliferation of the *Schizosaccharomyces* cell in the presence of the test compound to a level of proliferation of the *Schizosaccharomyces* cell in the absence of the test compound, wherein an increase in the level of proliferation in the presence of the test compound is indicative of inhibition of the cdc25 phosphatase by the test compound.
- 25
33. The assay of claim 32, wherein the *Schizosaccharomyces* cell is an *Schizosaccharomyces pombe* cell.
34. The assay of claim 32, wherein the *Schizosaccharomyces* cell is a conditional wee phenotype.
- 30
35. The assay of claim 34, wherein the *Schizosaccharomyces* cell is a *wee1-50* mutant.
36. The assay of claim 32, wherein the impairment of the wee1 protein kinase activity is caused by the overexpression of a nim1 activator in the *Schizosaccharomyces* cell.
- 35
37. The assay of claim 36, wherein the *Schizosaccharomyces* cell is an OP-nim1 mutant.

38. The assay of claim 32, wherein the *cdc25* phosphatase activity is a recombinant gene product expressed in the *Schizosaccharomyces* cell, and the *Schizosaccharomyces* cell lacks a functional endogenous *cdc25* phosphatase activity.
- 5 39. The assay of claim 38, wherein the *cdc25* phosphatase activity is a human *cdc25* or homolog thereof.
- 10 40. The assay of claim 39, wherein the human *cdc25* is selected from a group consisting of *cdc25A*, *cdc25B* and *cdc25C*.
41. The assay of claim 38, wherein the *cdc25* phosphatase activity is a human pathogen *cdc25* or homolog thereof.
- 15 42. The assay of claim 39, wherein the human pathogen is a fungus implicated in a mycotic infection selected from a group consisting of candidiasis, aspergillosis, mucormycosis, blastomycosis, geotrichosis, cryptococcosis, chromoblastomycosis, coccidioidomycosis, conidiosporosis, histoplasmosis, maduromycosis, rhinosporidiosis, nocardiosis, para-actinomycosis, penicilliosis, monoliasis, and sporotrichosis.
- 20 43. A *Schizosaccharomyces* cell comprising
- 25 i). an expressible recombinant gene encoding an exogenous *cdc25* phosphatase; and
- 30 ii). a conditionally impairable *wee1* protein kinase which can cause inhibition of cell proliferation by facilitating premature entry of the cell into mitosis under conditions wherein the *wee1* protein kinase is impaired, the premature entry into mitosis being mediated at least in part by the exogenous *cdc25* phosphatase and a reduced level of inhibitory phosphorylation of a *cdc2* protein kinase by the impaired *wee1* protein kinase.
44. The *Schizosaccharomyces* cell of claim 43, wherein the exogenous *cdc25* phosphatase comprises a human *cdc25* phosphatase.
- 35 45. The *Schizosaccharomyces* cell of claim 43, wherein the human *cdc25* phosphatase is selected from a group consisting of *cdc25A*, *cdc25B*, and *cdc25C*.

46. The *Schizosaccharomyces* cell of claim 43, wherein the recombinant cdc25 phosphatase is a human pathogen cdc25 or homolog thereof.
- 5 47. The *Schizosaccharomyces* cell of claim 46, wherein the human pathogen cdc25 is a cdc25 phosphatase of a fungus implicated in a mycotic infection.
48. The *Schizosaccharomyces* cell of claim 43, wherein the wee1 protein kinase is temperature sensitive and is impaired at a temperature above a permissive
10 temperature.
49. The *Schizosaccharomyces* cell of claim 48, wherein the *Schizosaccharomyces* cell is a *wee1-50* mutant.
- 15 50. The *Schizosaccharomyces* cell of claim 43, further comprising an overexpressed nim1 gene product which impairs the wee1 protein kinase.
51. The *Schizosaccharomyces* cell of claim 50, wherein the *Schizosaccharomyces* cell is an OP-nim1 mutant.
20
52. An anti-proliferative agent identified in the assay of claim 1.
53. A therapeutic composition comprising an anti-proliferative agent identified in the assay of claim 1.
25
54. A method of inhibiting proliferation of a cell comprising contacting the cell with an anti-proliferative agent identified in the assay of claim 1 in an amount sufficient to inhibit proliferation of the cell.
- 30 55. A cdc25 phosphatase inhibitor identified in the assay of claim 12.
56. A therapeutic composition comprising a cdc25 phosphatase inhibitor identified in the assay of claim 12.
- 35 57. A method of inhibiting proliferation of a cell comprising contacting the cell with a cdc25 phosphatase inhibitor identified in the assay of claim 12 in an amount sufficient to inhibit mitosis in the cell.

Figure 1

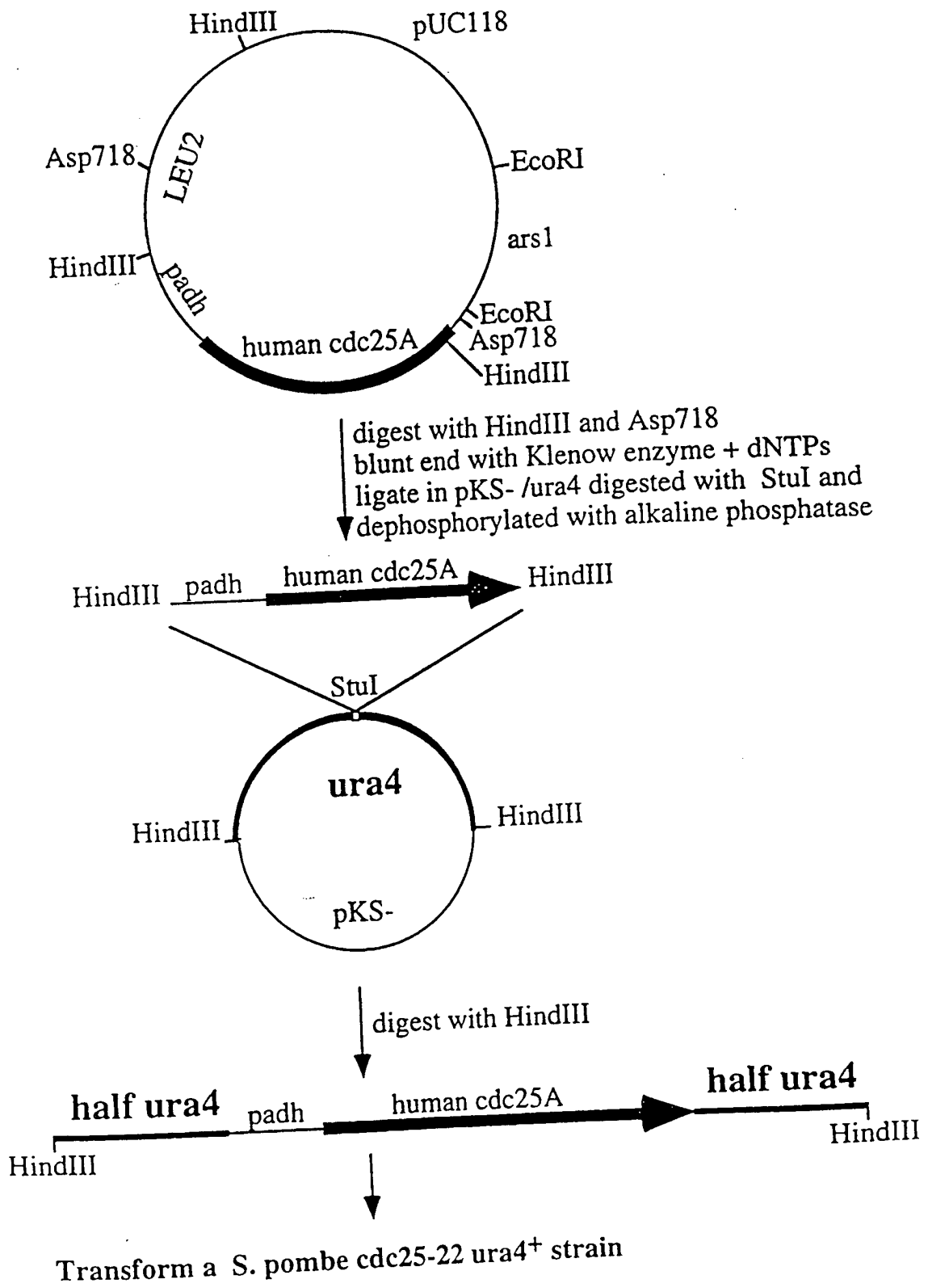


Figure 2

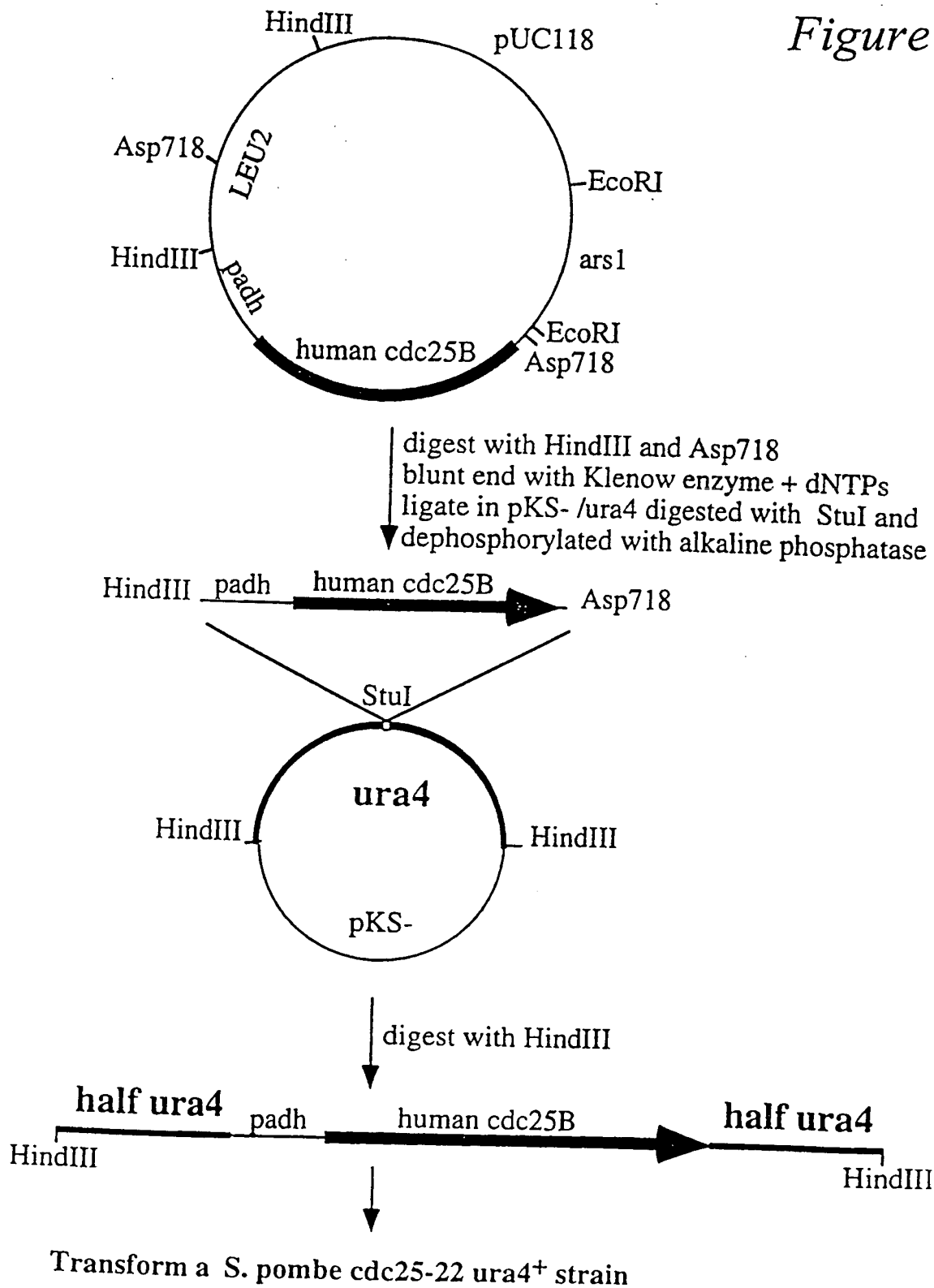


Figure 3

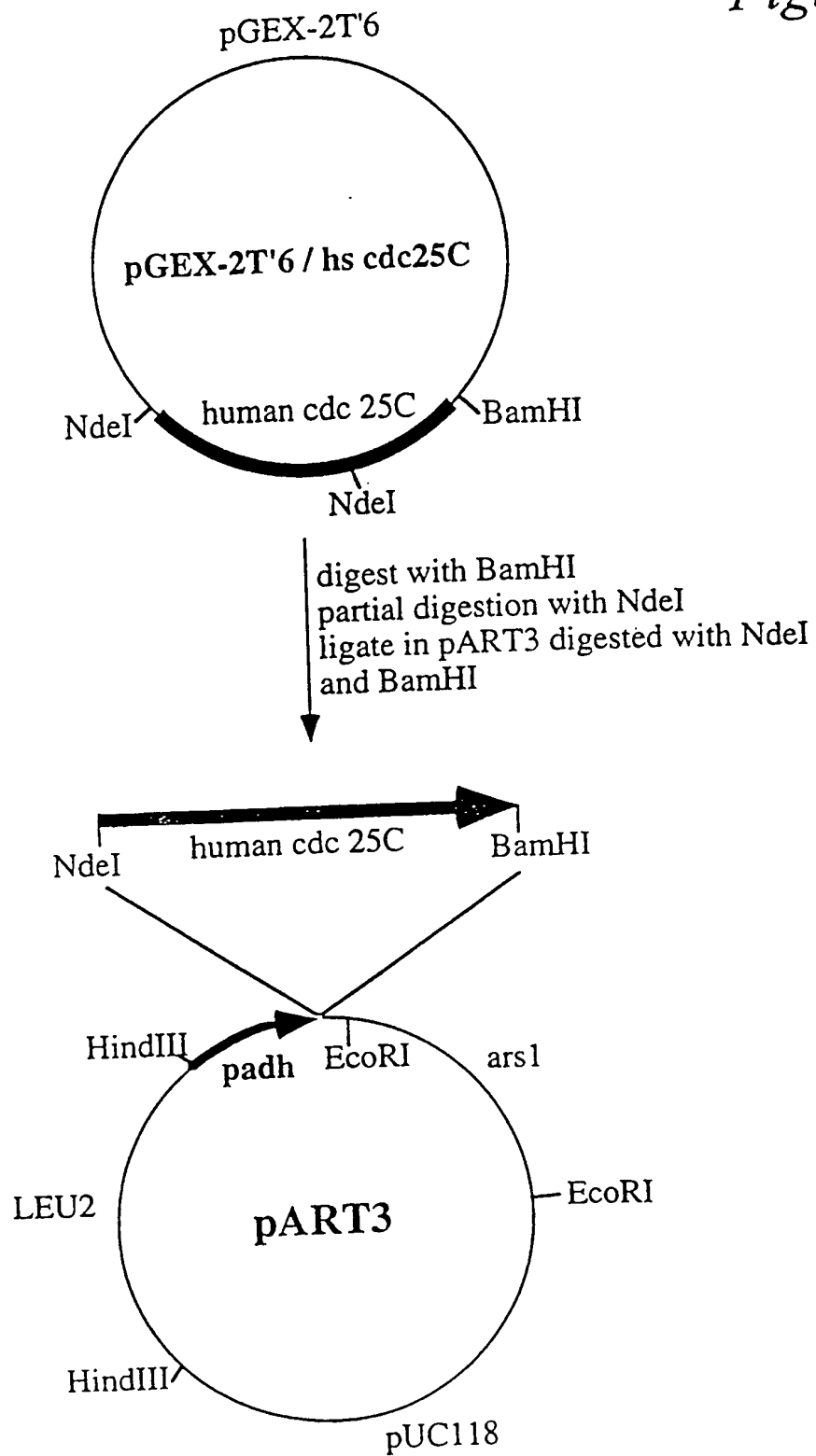
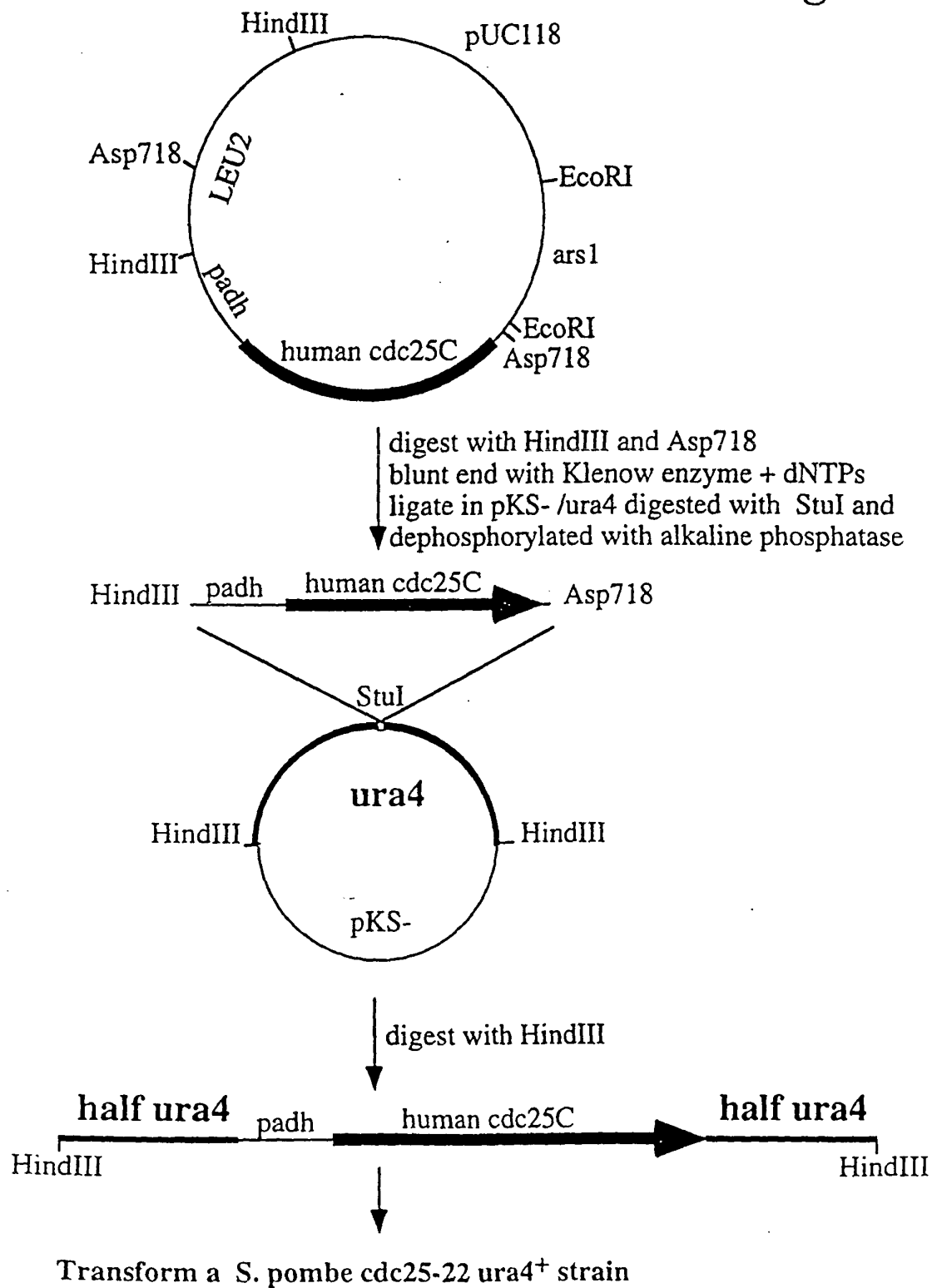


Figure 4



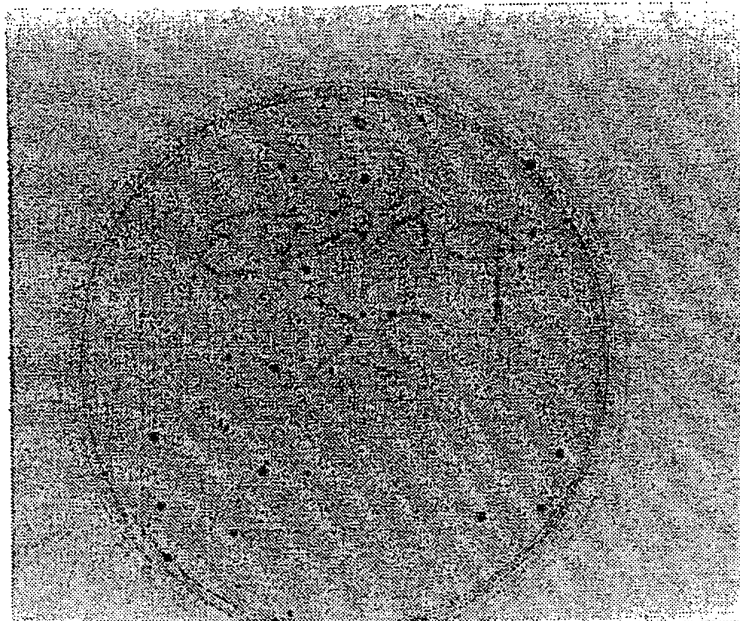


FIG. 5A

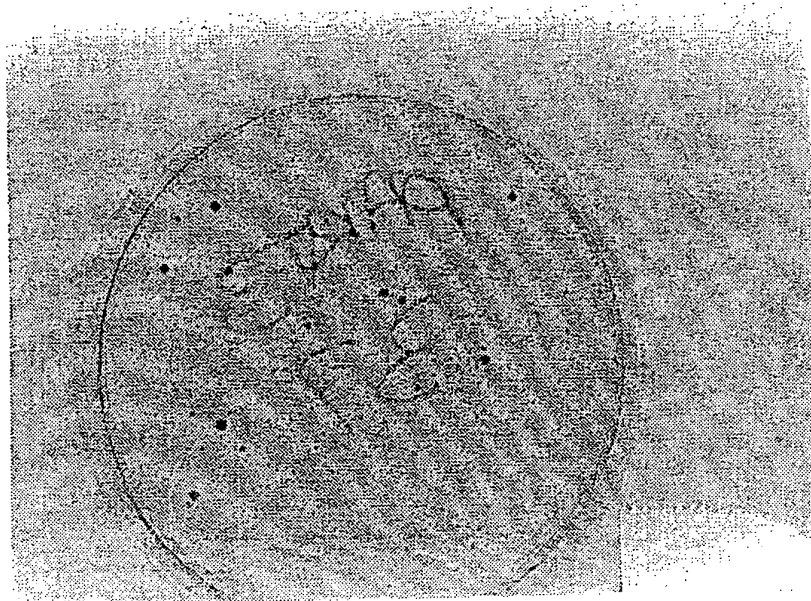


FIG. 5B

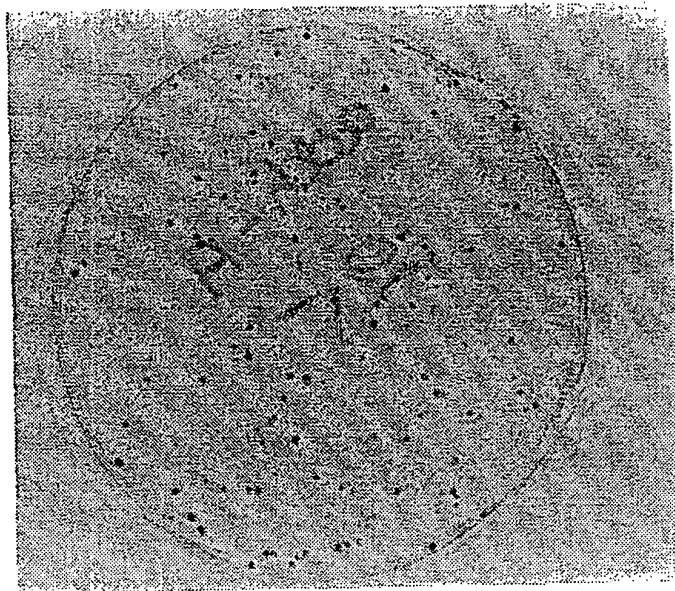


FIG. 6A

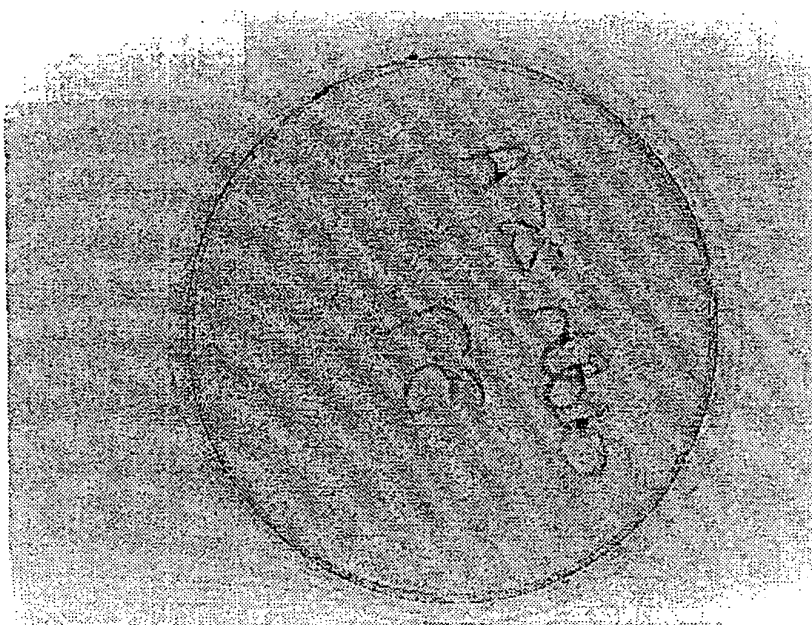


FIG. 6B

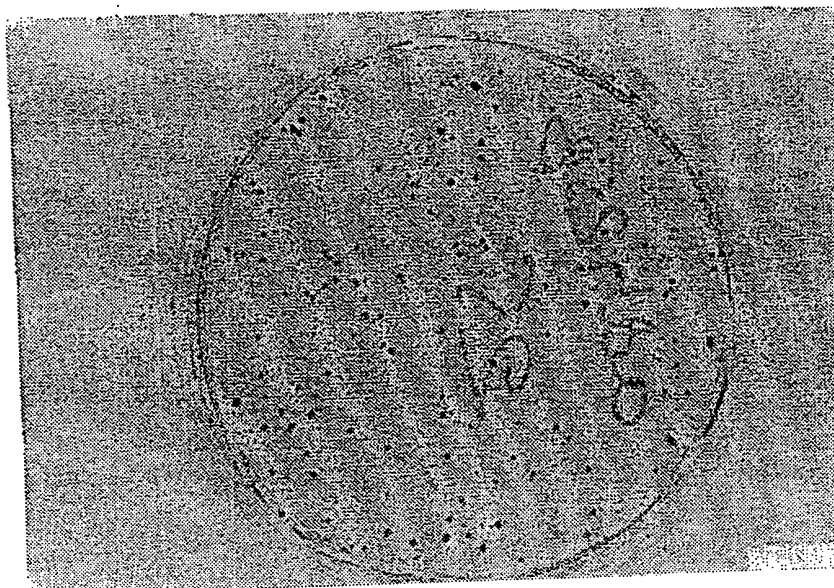


FIG. 7A

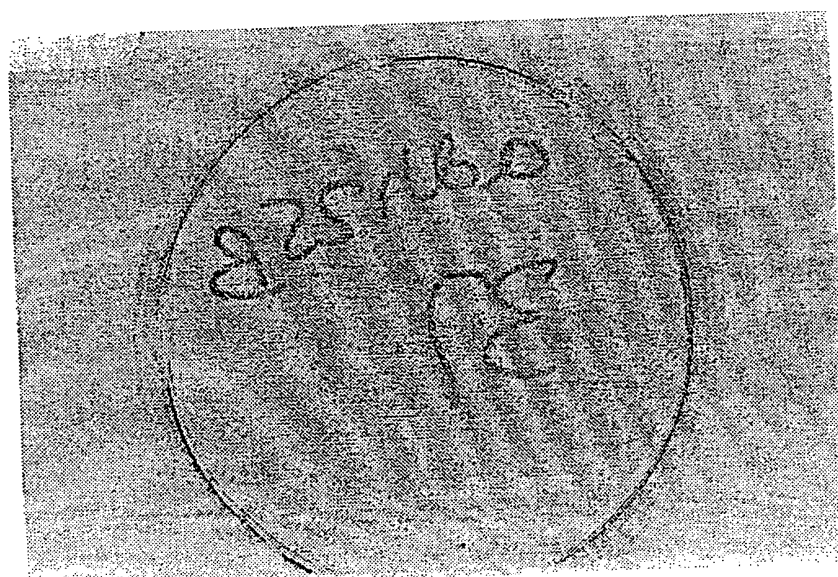


FIG. 7B

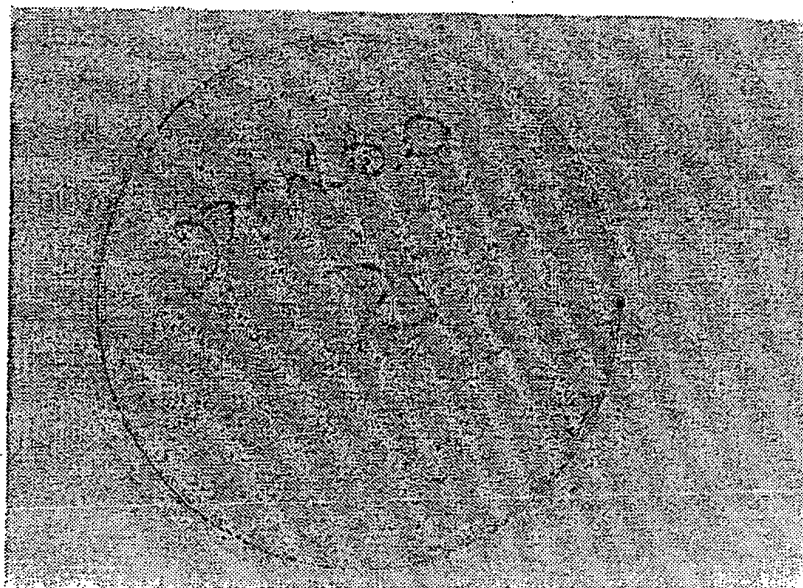


FIG. 8A

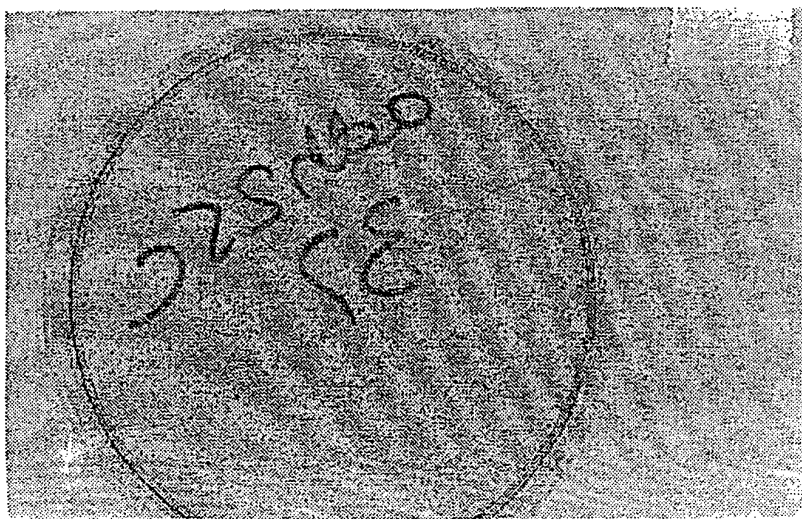


FIG. 8B

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/06365

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) : A61K 37/00; C07K 15/00; C12N 1/15; C12Q 1/18, 1/68

US CL : 435/6, 7.31, 32, 254.11; 514/12; 530/350

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 7.31, 32, 254.11; 514/12; 530/350

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	Cell, Vol. 67, issued 20 December 1991, Galaktionov et al., "Specific activation of cdc25 tyrosine phosphatases by B-type cyclins: evidence for multiple roles of mitotic cyclins", pages 1181-1194, see the entire document.	1, 2, 4, 28, 30, 31 ----- 5-7, 52-54
X --- Y	Cell, Vol. 49, issued 22 May 1987, Russell et al., "The mitotic inducer nim1 + functions in a regulatory network of protein kinase homologs controlling the initiation of mitosis", pages 569-576, see the entire document.	3, 8, 9, 11-15, 18-20, 32-35 ----- 5-7, 55-57
A	Cell, Vol. 67, issued 04 October 1991, Dunphy et al., "The cdc25 protein contains an intrinsic phosphatase activity", pages 189-196.	1-57

☒ Further documents are listed in the continuation of Box C.
 ☐ See patent family annex.

* Special categories of cited documents:	*T	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be of particular relevance	*X*	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
E earlier document published on or after the international filing date	*Y*	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubt on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*Z*	document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means		
P document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

14 JULY 1994

Date of mailing of the international search report

04 AUG 1994

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/US94/06365

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Cell, Vol. 67, issued 04 October 1991, Gautier et al., "cdc25 is a specific tyrosine phosphatase that directly activates p34cdc2", pages 197-211.	1-57
A	Nature, Vol. 359, issued 15 October 1992, Murray, "Creative blocks: cell-cycle checkpoints and feedback controls", pages 599-604.	1-57

B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

APS, Dialog

search terms: antimitotic, antimeiotic, anti-mitotic, anti-meiotic, mitosis, meiosis, assay, test, detect, mutant, mutation, cdc2, cdc25

